THE UNIVERSITY *of* EDINBURGH

Title	Evaluation of in vitro models for detection of hepatocyte toxicity induced by anti-cancer drugs
Author	Reid, Eilidh M.
Qualification	PhD
Year	2008

Evaluation of *in vitro* models for detection of hepatocyte toxicity induced by anti-cancer drugs

Eilidh Reid

A thesis submitted for the degree of Doctor of Philosophy (PhD) in the College of Medicine and Veterinary Medicine

The University of Edinburgh
April 2007

Declaration

The work presented in this thesis was carried out at the Cancer Research Centre, University of Edinburgh. In accordance with the regulations of the University, I declare that this thesis has been composed entirely by myself and that the work presented is my own except where I have indicated the contribution of others.

This work has not been submitted for any other degree or professional qualification.

Eilidh Reid

04 April 2007

Acknowledgments

Firstly, I would like to thank my supervisors, Dr. Sylvie Guichard and Professor Duncan Jodrell, for providing me with the opportunity to undertake a PhD in the Cancer Research Centre. I would also like to thank them very much for their helpful input and support during my PhD. Funding from a University of Edinburgh PhD scholarship made it possible for me to undertake the work in the laboratory, and I am grateful to the University for providing excellent facilities in which to work and learn.

Some of the work presented in this thesis would not have been possible without the help and advice of the United Kingdom Human Tissue Bank (UKHTB), and in particular I would like to mention Dr Shaun Kingston for his advice at the end of a telephone, and the administration staff for making all the necessary arrangements to allow efficient transport of cells for my research.

I would like to thank other members of the University of Edinburgh Cancer Research UK Centre in which I carried out the lab work. I'd like to thank Janet Macpherson and Kenny Macleod for their guidance when working with RNA. In the lab, Rhona Aird carried out the RM175 and HC11 work in the non-hepatoma cell lines. And I would like to thank everyone else for providing daily entertainment and plenty of socialising!

And finally, much thanks goes to my family, Kenny Alison and Alistair, and my now fiancé Iain: Not only for making it possible for me to study by helping with the financial side of things, but mainly for their continued support and encouragement.

Abstract

Drug-induced hepatotoxicity is a major cause of failure of drug candidates during development and of drug withdrawal post-marketing. Exposure of individuals to many different classes of drugs, including several anti-cancer agents, can result in liver toxicity. In oncology, the aim of treatment with cytotoxic drugs is to selectively target and kill all cancerous cells, whilst leaving the healthy tissue unharmed. In practice, anti-cancer drugs induce cytotoxic effects in all proliferating cells and, to a certain extent, non-dividing cells. For some anti-cancer drugs this manifests as liver-related dose-limiting toxicity. If this occurs in early phase clinical trials, it may stop clinical development of that drug. Unfortunately, current pre-clinical models poorly predict a potentially hepatotoxic anti-cancer agent.

Therefore, the aim of the studies included in this thesis is to evaluate *in vitro* models in which anti-cancer drug-induced hepatotoxicity may be detected, to decrease the likelihood of hepatotoxic anticancer agents entering clinical development. *In vitro* models that represent hepatocytes, the major cell type in the liver, were selected, and were all of human origin. Murine models were not considered as there are significant inter-species differences that may impact on drug metabolism and therefore on drug-induced liver toxicity. The selected models comprised fresh and cryopreserved hepatocytes (from 24 and 3 individuals respectively), an immortalised hepatocyte cell line (THLE-2) and hepatoma cell lines (Hep 3B2.1-7, Hep G2, Huh-7D12, PLC/PRF/5, and SK-HEP-1). These cells were characterised, and then assessed in terms of their sensitivity to a known hepatotoxic drug (mithramycin), drugs used in clinical practice in colorectal cancer (oxaliplatin, 5-FU) and novel agents in preclinical development (ruthenium compounds).

In order to characterise the cell models, morphology, growth parameters, and viability were considered. Fresh and cryopreserved hepatocytes did not proliferate in culture, whereas the cell lines had doubling times ranging from 33 to 54 hours. Hepatocyte viability varied between donors at isolation and after transport. The impact of transporting fresh hepatocytes in different cold-storage media, and the duration between isolation and plating, on hepatocyte viability was investigated.

Gene expression was also studied by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The genes evaluated were involved in drug transport (ABCB1), drug metabolism (CYP450s, UGTs), or were liver specific genes (albumin, transthyretin, α-1-antitrypsin). mRNA expression of all liver-specific genes except CYP1A1 was higher in fresh hepatocytes than in any of the other models investigated. None of the *in vitro* models mimic the gene expression profile of hepatocytes but the hepatoma cell lines express some liver-specific genes. Stress-induced genes (p21, heat-shock protein 70 and hypoxia-inducible factor-1α) were evaluated to identify the impact of hepatocyte plating on stress gene expression. mRNA expression of all stress-induced genes increased following plating in the majority of donors. The impact on gene expression of the duration of transport, the transport media, and plating, was assessed to improve the use of fresh human cells in hepatotoxicity assessment.

To identify the most appropriate *in vitro* model on the basis of the identification of hepatocyte-toxic drugs, cytotoxicity assays were carried out following exposure of each of the cell models to increasing concentrations of the selected drugs. DNA damaging agents induce cytotoxic effects; therefore cell death was considered as the endpoint for evaluating drug-induced hepatotoxicity. The sulforhodamine B and MTT cytotoxicity assays were carried out, followed by determination of IC_{50} values from the data obtained. Instead of aiming for an absence of toxicity, it is more interesting to determine the difference between an active concentration killing tumour cells and achievable in the clinic, and a toxic concentration, killing hepatocytes. This "*in vitro* therapeutic index" may be able to discriminate which drug is likely to induce significant liver toxicity, at predicted therapeutic doses. Colon cancer lines were used to determine the active concentrations for mithramycin, oxaliplatin and 5-FU. The effect of these drugs was then determined in the hepatocyte models. The "*in vitro* therapeutic index" was able to discriminate between known hepatotoxin mithramycin, and oxaliplatin and 5-FU which both had a higher therapeutic index in fresh hepatocytes. Hepatoma cell lines gave lower therapeutic indices but the ranking of the compounds was similar as with the fresh hepatocytes. Finally, using this approach, the hepatotoxicity of two ruthenium

compounds in pre-clinical development (RM175 & HC11) was identified, using fresh hepatocytes and hepatoma cell lines.

A subset of *in vitro* models (fresh and cryopreserved hepatocytes, Hep3B2.1-7, HepG2, Huh-7D12 and PLC/PRF/5), which appeared to be the most promising based on gene expression and cytotoxicity data, were then selected to study the contribution of oncosis and/or apoptosis to cytotoxicity, and to see whether a hepatotoxic drug could be detected earlier during drug exposure. Assays to measure ATP levels and capsase-3/7 activation in drug-treated cells were optimised and used for mithramycin-, oxaliplatin- and 5-FU-treated cells. ATP levels and caspase-3/7 activation varied between cell lines and hepatocytes in response to all three drugs investigated, and was detectable immediately following drug treatment.

These data suggest that fresh hepatocytes are a suitable *in vitro* model in which to accurately identify hepatotoxicity associated with anti-cancer drugs. They express liver-specific genes, and are more sensitive to the hepatotoxic drug mithramycin, than to oxaliplatin and 5-FU. A cytotoxicity assay using hepatoma cell lines (Hep3B2.1-7 and Huh-7D12) could also be used, despite their low expression of liver-related genes. These results should help select the most appropriate *in vitro* models to detect hepatotoxic anticancer agents in the future.

Contents

Declaration ... ii
Acknowledgements ... iii
Abstract ... iv
Table of contents ... vii
List of figures ... xi
List of tables ... xiii
List of abbreviations ... xiv

CHAPTER 1: Introduction ... 1
1.1 THE LIVER ... 2
1.1.1 Structure of the liver ... 2
1.1.1.1 Hepatocytes ... 4
1.1.1.2 Cholangiocytes ... 5
1.1.1.3 Kupffer Cells ... 5
1.1.2 Functions of the liver ... 6
1.1.2.1 Protein Synthesis ... 6
1.1.2.2 Carbohydrate and Lipid Metabolism ... 7
1.1.2.3 Drug Metabolism and Transport ... 7
1.1.2.3.1 Phase I Reactions ... 9
1.1.2.3.1.1 Cytochrome P450s activity ... 10
1.1.2.3.1.2 Cytochrome P450 Regulation ... 11
1.1.2.3.2 Phase II Reactions ... 17
1.1.2.3.3 Drug uptake and excretion ... 19
1.1.2.3.4 Factors affecting drug metabolism and excretion ... 22
1.1.3 Liver Function Tests ... 24
1.2 HEPATOTOXICITY ... 28
1.2.1 Types of Liver Damage Caused by Drugs ... 29
1.2.1.1 Dose-dependent ... 30
1.2.1.2 Idiosyncratic ... 30
1.2.2 Mechanisms of Liver Damage Caused by Drugs ... 31
1.2.2.1 Apoptosis ... 37
1.2.2.2 Oncosis ... 40
1.2.3 Risk Factors for Hepatotoxicity ... 40
1.2.3.1 General Risk Factors ... 40
1.2.3.2 Risk Factors for Hepatotoxicity in Patients with Cancer ... 42
1.2.4 Testing for Drug-Induced Hepatotoxicity ... 44
1.2.4.1 Isolated enzymes, organelles, and subcellular fractions ... 46
1.2.4.2 Isolated cells in vitro ... 46
1.2.4.3 Liver slices ... 49
1.2.4.4 Isolated perfused liver ... 50
1.2.4.5 Liver in vivo ... 50
1.2.4.6 Clinical Trials ... 50
1.2.4.7 Testing for Drug-Induced Hepatotoxicity in Oncology ... 51
1.3 HEPATOTOXICITY INDUCED BY ANTICANCER AGENTS ... 52
1.3.1 Chemotherapy-induced hepatotoxicity ... 53
1.3.1.1 Type of chemotherapy (single-agents) ... 53
1.3.1.2 Route of administration ... 56
1.3.2 Hepatotoxicity during clinical development of novel anti cancer agents ... 57
1.4 AIMS AND OBJECTIVES OF THIS STUDY ... 60

CHAPTER 2: Results: Characterisation and Optimisation of Cell Models ... 62
2.1 INTRODUCTION ... 63
2.2 CELL MORPHOLOGY ... 63
2.2.1 Hepatocytes ... 63
2.2.2 Immortalised hepatocyte cell line ... 66
2.2.3 Hepatoma cell lines ... 66

2.3 VIABILITY OF HEPATOCYTES **68**
2.3.1 Fresh human hepatocytes 68
2.3.1.1 Initial viability and loss of viability during transport 68
2.3.1.2 Viability during culture 69
2.3.2 Cryopreserved human hepatocytes 71
2.4 CELL GROWTH CHARACTERISTICS **72**
2.4.1 Hepatoma cell lines and immortalised hepatocytes 72
2.5 GENE EXPRESSION OF IN VITRO MODELS **74**
2.5.1 Quantitative RT-PCR set-up 76
2.5.2 Reference Gene Selection 78
2.5.3 Expression of liver-specific genes in fresh hepatocytes 80
2.5.4 Expression of liver-specific genes in cryopreserved hepatocytes 84
2.5.5 Expression of liver-specific genes in immortalised and hepatoma cell lines 85
2.5.6 Comparison of gene expression between in vitro models 87
2.5.7 Expression of stress-induced genes in hepatocytes 89
2.5.7.1 HIF1A, p21 and HSP-70 mRNA expression in fresh hepatocytes 89
2.5.7.2 Stress-induced genes in cryopreserved hepatocytes 94
2.5.7.3 Effect of stress gene expression on hepatocyte viability and liver-specific gene expression 95
2.6 OPTIMISATION OF FRESH HEPATOCYTE TRANSPORT **96**
2.6.1 Impact of transport media on hepatocyte viability 99
2.6.2 Impact of transport time on hepatocyte viability 100
2.6.3 Impact of transport time on hepatocyte gene expression 101
2.6.4 Impact of transport media on hepatocyte gene expression 104
2.7 DISCUSSION **107**
2.7.1 Hepatocyte Viability, Attachment, and Culture 109
2.7.2 Cell Proliferation 111
2.7.3 Liver gene expression 111
2.7.4 Stress gene expression 114
2.7.5 Comparison of fresh hepatocyte transport media 115
2.7.6 Conclusions 116

CHAPTER 3: Results Assessment of liver toxicity: Cytotoxicity of selected anti-cancer drugs *in vitro* **118**
3.1 INTRODUCTION **119**
3.1.1 *In vitro* Cytotoxicity Assays 120
3.1.1.1 SRB Assay 120
3.1.1.2 MTT Assay 120
3.1.2 Pharmacological Properties and Hepatotoxic Potential of Selected Drugs 121
3.1.2.1 5-fluorouracil 121
3.1.2.2 Oxaliplatin 122
3.1.2.3 Mithramycin 123
3.1.2.4 Ruthenium compounds HC11 and RM175 124
3.1.3 Principle of Therapeutic Index 125
3.2 *IN VITRO* ASSAYS **126**
3.2.1 Determination of Optimal Time-point to Detect Cytotoxicity 127
3.2.2 Comparison of SRB and MTT assays 128
3.3 DRUG TREATMENT OF CELLS **129**
3.3.1 Cytotoxicity in Colon Cancer Cell Lines 129
3.3.2 Cytotoxicity in *in vitro* liver Models 133
3.3.2.1 Cytotoxicity in hepatoma and immortalised hepatocytes cell lines 133
3.3.2.2 Cytotoxicity in cryopreserved hepatocytes 135
3.3.2.3 Cytotoxicity in fresh hepatocytes 135
3.3.3 Therapeutic Index 138
3.4 POTENTIAL INFLUENCES ON CYTOTOXICITY IN FRESH HEPATOCYTES **140**
3.4.1 Donor Demographics 140
3.4.2 Gene Expression 141
3.5 KINETICS OF CELL DEATH **144**

3.6 APPLICATION OF *IN VITRO* HEPATOTOXICITY TESTING TO DRUGS IN DEVELOPMENT **147**
3.6.1 Ruthenium Compounds: HC11 and RM175 147
3.7 DISCUSSION **149**
3.7.1 *In vitro* cytotoxicity assays 149
3.7.2 Drugs tested and Therapeutic Index 151
3.7.3 Influences on Cytotoxicity in Fresh Hepatocytes 155
3.7.4 Time-points selected and Kinetics of cell death 156
3.7.5 Conclusions 156

CHAPTER 4: Results: Earlier detection of cell stress induced by anti-cancer agents in selected in vitro models of the liver **158**
4.1 INTRODUCTION **159**
4.1.1 Methods Available for Detection of Cell Death 160
4.1.2 Chapter Aims and Overview 163
4.2 ESTABLISHMENT OF THE SELECTED IN VITRO ASSAYS **164**
4.2.1 Adenylate Nucleotide Ratio Assay 164
4.2.2 Caspase-3/7 Assay 169
4.3 ATP LEVELS IN CELLS FOLLOWING DRUG TREATMENT **171**
4.3.1 ATP Levels in Cells Following Mithramycin Treatment 171
4.3.2 ATP Levels in Cells Following Oxaliplatin Treatment 173
4.3.3 ATP Levels in Cells Following 5-FU Treatment 175
4.4 CASPASE-3/7 ACTIVATION IN CELLS FOLLOWING DRUG TREATMENT **178**
4.4.1 Caspase-3/7 Activation Following Mithramycin Treatment 178
4.4.2 Caspase-3/7 Activation Following Oxaliplatin Treatment 181
4.4.3 Caspase-3/7 Activation Following 5-FU Treatment 183
4.5 COMPARISON OF EARLY (24-HOUR) AND LATER (96-HOUR) DATA **185**
4.5.1 Mithramycin 186
4.5.2 Oxaliplatin 187
4.5.3 5-FU 187
4.6 DISCUSSION **192**
4.6.1 Adenylate Nucleotide Ratio Assay and Results 192
4.6.2 Caspase-3/7 Assay and Results 194
4.6.3 Mechanisms of Cell Death in Hepatocyte Models 195
4.6.4 Earlier Detection of Cell Death 196
4.6.5 Conclusions 196

CHAPTER 5: Conclusions and future directions **198**
5.1 SUMMARY AND CONCLUSIONS **198**
5.2 FUTURE DIRECTIONS **201**

CHAPTER 6: Materials and Methods **204**
6.1 MATERIALS **205**
6.1.1 Cell Culture 205
6.1.1.1 Primary Cells 205
6.1.1.2 Cell Lines 205
6.1.1.3 Cell Culture Materials 205
6.1.2 Cytotoxicity Assays 206
6.1.3 RNA Extraction and qRT-PCR 207
6.1.4 Protein Isolation and Western Blotting 208
6.2 METHODS: CELL CULTURE **208**
6.2.1 Maintenance of Cell Lines 208
6.2.2 Cell Harvesting 209
6.2.3 Cell Counting 209
6.2.4 Cryopreservation and Recovery of Cells from Liquid Nitrogen 212
6.2.5 Growth Characterisation of Proliferating Cell Lines 212
6.2.6 Fresh Hepatocyte Cell Culture 216
6.2.7 Counting & Assessment of Hepatocyte Viability by Trypan Blue Exclusion 216

6.2.8 Cryopreserved Human Hepatocyte Cell Culture 217
6.3 METHODS: IN VITRO STUDIES 223
6.3.1 Drugs 223
6.3.2 Sulforhodamine B (SRB) Assay 223
6.3.3 MTT Assay 224
6.3.3.1 Determination of optimal MTT concentration 224
6.3.4 Cytotoxicity Studies with Hepatocytes 226
6.3.4.1 Fresh hepatocytes 226
6.3.4.1 Cryopreserved hepatocytes 226
6.3.5 Analysis of Cytotoxicity Data 226
6.3.6 Detection of the Mechanism of Cell Death 227
6.3.6.1 ATP and ADP Assays 227
6.3.6.2 Caspase-3/7 Activity Assay 232
6.3.6.3 Data analysis 233
6.4 METHODS: RNA EXTRACTION AND qRT-PCR 234
6.4.1 RNA Extraction 234
6.4.2 DNase Treatment of RNA Samples 234
6.4.3 Quantification of RNA by NanoDrop 235
6.4.4 Assessment of RNA Integrity 235
6.4.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) 239
6.4.6 Size of qRT-PCR Products 243
6.4.7 Statistical Analysis of qRT-PCR 243
6.5 METHODS: PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS 244
6.5.1 Protein Isolation from Tri Reagent 244
6.5.2 Bicinchoninic Acid Protein (BCA) Assay 244
6.5.3 Western Blotting 245

References 247

Appendix A: Published Papers 276
Guichard, S., Else, R., **Reid, E.**, Zeitlin, B., Aird, R., Muir, M., Dodds, M., Fiebig, H., Sadler, P., Jodrell, D. (2006) Anti-tumour activity in non-small cell lung cancer models and toxicity profiles for novel ruthenium(II) based organo-metallic compounds. Biochemical Pharmacology 71(4):408-415 277

Guichard, S.M., Macpherson, J.S., Mayer, I., **Reid, E.**, Muir, M., Dodds, M., Alexander, S., Jodrell, D.I. (2007) Gene expression predicts differential capecitabine metabolism, impacting on both pharmacokinetics and antitumour activity. European Journal of Cancer doi:10.1016/j.ejca.2007.10.023 286

List of figures

Figure 1.1: Schematic representation of the fine structure of the human liver..... 3
Figure 1.2: Catalytic cycle of Cytochrome P450.....11
Figure 1.3: Receptor-dependent CYP induction in hepatocytes..... 15
Figure 1.4: Mechanisms of Liver Injury..... 34
Figure 1.5: Death-receptor-dependent and –independent pathways in anti-cancer drug-induced apoptosis..... 39

Figure 2.1: Morphological comparison of fresh hepatocytes in living monolayers, 12- and 108- hours after plating..... 65
Figure 2.2: Morphological comparison of attached cryopreserved hepatocytes in living monolayers..... 65
Figure 2.3: Immortalised hepatocyte cell line, THLE-2..... 66
Figure 2.4: Morphological comparison of hepatoma cell lines in living monolayers..... 67
Figure 2.5: Cell viability determined by MTT assay over time in culture..... 70
Figure 2.6: CYP3A4 product melt curve (A) and rotorgene cycling (B)..... 76
Figure 2.7: Comparison of reference genes expression in hepatocytes..... 79
Figure 2.8: Gene expression in fresh human hepatocytes..... 83
Figure 2.9: Gene expression in cryopreserved human hepatocytes..... 84
Figure 2.10: Gene expression in 5 hepatoma cell lines and immortalised hepatocyte cell line THLE-2.....86
Figure 2.11: Gene expression in hepatocytes before and after plating, cryopreserved hepatocytes and hepatoma cell lines..... 88
Figure 2.12: HIF1A mRNA expression in fresh hepatocyte samples..... 91
Figure 2.13: p21 mRNA expression in fresh hepatocyte samples..... 92
Figure 2.14: HSP-70 mRNA expression in fresh hepatocyte samples..... 93
Figure 2.15: HIF1A, HSP70 and p21 expression in cryopreserved human hepatocytes.... 94
Figure 2.16: Correlation between HSP-70 mRNA expression and hepatocyte viability..... 95
Figure 2.17: Impact of initial hepatocyte viability on viability after transport..... 96
Figure 2.18: Impact of initial hepatocyte viability on viability after transport..... 98
Figure 2.19: Impact of transport media on hepatocyte viability..... 99
Figure 2.20: Time in transport vs. loss of hepatocyte viability..... 100
Figure 2.21: Influence of transport time on CYP1A1, CYP3A4 and UGT2B* expression.... 103
Figure 2.22: Gene expression before and after transport in Celsior, DMEM, and UW.....105
Figure 2.23: Gene expression before and after transport in Celsior, DMEM, and UW.....106

Figure 3.1: Chemical structure of 5-FU..... 121
Figure 3.2: Chemical structure of oxaliplatin.....123
Figure 3.3: Chemical structure of mithramycin..... 124
Figure 3.4: Chemical Structure of RM175 and HC11.....125
Figure 3.5: Time-course of cytotoxicity in PLC/PRF/5 and fresh hepatocytes..... 128
Figure 3.6: Comparison of MTT and SRB assays.....129
Figure 3.7: 24-hour 5-FU treatment of colon cancer cell line HCT-15..... 130
Figure 3.8: IC_{50} values in colorectal cancer cell lines..... 132
Figure 3.9: Spread of IC_{50}s in mithramycin, oxaliplatin and 5-FU–treated cell lines..... 134
Figure 3.10: IC_{50}s in mithramycin, oxaliplatin and 5-FU–treated hepatocytes..... 136
Figure 3.11: Range of IC_{50} in all cell models.....137
Figure 3.12: Therapeutic Index.....139
Figure 3.13: Kinetics of cell death in hepatoma cell lines after drug treatment..... 146
Figure 3.14: Kinetics of cell death in fresh hepatocytes after drug treatment..... 147
Figure 3.15: Cytotoxicity of RM175 (A) and HC11 (B) in 4 hepatoma cell lines using the SRB assay.....148
Figure 3.16: Cytotoxicity of HC11 (left) and RM175 (right) in fresh hepatocytes..... 148
Figure 3.17: 'Therapeutic index' of 5 anti-cancer agents..... 148

Figure 4.1: Summary of methods available for detection of apoptosis or oncosis.....161
Figure 4.2: ADP and ATP levels in HepG2 cells after heat-shock 166

Figure 4.3: Oncosis induction by Kahalalide F in HepG2 cells 168
Figure 4.4: ADP and ATP levels in HCT116 cells after TRAIL exposure 169
Figure 4.5: Induction of apoptosis in HCT116 cells treated with TRAIL 170
Figure 4.6: Caspase-3/7 activity in HepG2 cells exposed to kahalalide F 170
Figure 4.7: ATP levels following mithramycin treatment in hepatoma cell lines 172
Figure 4.8: ATP levels following mithramycin treatment in hepatocytes 173
Figure 4.9: ATP levels following oxaliplatin treatment in hepatoma cell lines 174
Figure 4.10: ATP levels following oxaliplatin treatment in hepatocytes 175
Figure 4.11: ATP levels following 5-FU treatment in hepatoma cell lines 176
Figure 4.12: ATP levels following 5-FU treatment in hepatocytes 177
Figure 4.13: Caspase-3/7 following mithramycin treatment in hepatoma cell lines 179
Figure 4.14: Caspase-3/7 levels following mithramycin treatment in hepatocytes 180
Figure 4.15: Caspase-3/7 levels following oxaliplatin treatment in hepatoma cell lines 182
Figure 4.16: Caspase-3/7 levels following oxaliplatin treatment in hepatocytes 183
Figure 4.17: Caspase-3/7 levels following 5-FU treatment of hepatoma cell lines 185
Figure 4.18: Caspase-3/7 levels following 5-FU treatment of hepatocytes 186
Figure 4.19: Comparison of ATP, SRB and MTT assay in fresh hepatocytes treated with mithramycin, oxaliplatin or 5-FU 189
Figure 4.20: Comparison of ATP, SRB and MTT assay in hepatoma cell lines treated with mithramycin 190
Figure 4.21: Comparison of ATP, SRB and MTT assay in hepatoma cell lines treated with oxaliplatin 191
Figure 4.22: Comparison of ATP, SRB and MTT assay in hepatoma cell lines treated with 5-FU 192

Figure 6.1: Calibration, and OD per density of HepG2 cells 214
Figure 6.2: Exponential growth of HepG2 cells 215
Figure 6.3: Determination of optimal MTT concentration for cytotoxicity assays 225
Figure 6.4: ATP and ADP Standard Curve 229
Figure 6.5: Kinetics of the ApoGlow™ assay 229
Figure 6.6: Comparison of Cambrex and Promega kit signal half-life 231
Figure 6.7: Caspase-3 and Caspase-7 standard curves 233
Figure 6.8: Agilent Assessment of RNA integrity 236
Figure 6.9: Verification of qRT-PCR product sizes 246

List of tables

Table 1.1: Drug metabolising enzymes and examples of their anti-cancer drug Substrates 9
Table 1.2: Regulation of drug metabolising enzymes and transporters 16
Table 1.3: Drug transporters and examples of their anti-cancer drug substrates 22
Table 1.4: Normal values of LFTs 25
Table 1.5: Grades of liver test abnormalities 27
Table 1.6: Effect of chemotherapy on LFTs and liver injury, when used as single agents ... 54
Table 1.7: New anti-cancer agents that have had effects on LFTs and/or caused liver injury during clinical trials 58

Table 2.1: Fresh hepatocyte viability at isolation and after transport 68
Table 2.2: Influence of donor demographics on cell viability 69
Table 2.3: Viability of cryopreserved hepatocyte donors before and after cryopreservation. 71
Table 2.4: Doubling time, plating efficiency & plating density per cell line (hepatomas, THLE-2) 73
Table 2.5: Genes of interest, reference genes, and references for information on each gene 75
Table 2.6: Standard curve parameters and melting temperature of qRT-PCR products 77
Table 2.7: Gene expression in cryopreserved hepatocytes 84
Table 2.8: Spearman correlation between time from resection and change in mRNA expression 102

Table 3.1: Colon cancer cell line 50% inhibitory concentrations (IC_{50}) 131
Table 3.2: Liver cell lines 50% inhibitory concentrations (IC_{50}) 134
Table 3.3: Cryopreserved hepatocyte cytotoxicity 135
Table 3.4: Fresh hepatocytes 50% inhibitory concentrations (IC_{50}) 136
Table 3.5: Spearman correlation of donor factors and IC_{50} values 140
Table 3.6: Mann Whitney test of donor sex or smoking status and IC_{50} values 141
Table 3.7: Spearman correlation of gene expression at start of drug treatment and IC_{50} values 143
Table 3.8: Spearman correlation of change in gene expression and IC_{50} values 143
Table 3.9: Selection of drug concentration for investigation of kinetics of cell death 144

Table 6.1: Source and details of cell lines used for in vitro studies 211
Table 6.2: Doubling time, plating efficiency & plating density of colon cancer cell lines 215
Table 6.3: Donor demographics of patients from which fresh hepatocytes were used for in vitro studies 218
Table 6.4: Donor demographics from which hepatocytes were used for transport media testing 220
Table 6.5: Donor demographics of patients from which cryopreserved hepatocytes were obtained 222
Table 6.6: RNA integrity numbers (RIN) obtained for RNA samples, using an Agilent 2100 Bioanalyser and Agilent software 237
Table 6.7: Primer sequences for qRT-PCR 241
Table 6.8: Primary antibodies for Western Blot detection 246
Table 6.9: Secondary antibodies for Western Blot detection 246

Abbreviations

°C	Degrees Celsius
%w/v	Percentage weight by volume
%v/v	Percentage volume by volume
5-FU	5-fluorouracil
18S	18S ribosomal RNA
ACTB	Actin B
ADP	Adenosine diphosphate
ADP-CR	Adenosine diphosphate-converting reagent
AFP	α-foetoprotein
ALB	Albumin
AMPS	Ammonium persulphate
Antibiotics	10000U/ml penicillin and 10000μg/ml streptomycin
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B2M	β-2-microglobulin
BCA	Bicinchoninic Acid
BEBM	Bronchial Epithelial Medium
bp	Base pair(s)
BSA	Bovine Serum Albumin
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNase	Deoxynuclease
dNTP	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
ECACC	European Collection of Cell Cultures
ECM	Extra-cellular matrix
EGF	Human epidermal growth factor
FCS	Foetal calf serum
g	G-force
g	Gram(s)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HIF1A	hypoxia-inducible factor 1, alpha subunit
hr	hour
HRP	Horseradish peroxidise
HSP-70	heat shock 70kDa protein 2
IC_{50}	50% Inhibitory concentration of drug
Ig	Immunoglobulin
kDa	kilodalton(s)
KHF	Kahalalide F
l	litre(s)
LFTs	Liver function tests
μ	micro
m	metre(s)
m	milli
M	molar
min(s)	minute(s)
mRNA	messenger RNA
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MW	Molecular weight
NaCl	sodium chloride
n	Nano
NCI	National Cancer Institute (American)
NCBI	National Centre for Biotechnology Information

NHS	National Health Service
NMR	Nucleotide monitoring reagent
NRR	Nucleotide releasing reagent
OD	Optical density
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
pH	power of hydrogen
PVDF	Polyvinylidene fluoride
RFU	relative fluorescence unit(s)
RLU	relative luminescence unit(s)
RNA	Ribonucleic acid
rpm	Rotations/revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
qRT-PCR	quantitative Real-time polymerase chain reaction
s	seconds
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SERPINA1	Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1
SRB	Sulforhodamine B
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TI	Therapeutic Index
TTR	Transthyretin (prealbumin, amyloidosis type 1)
TRAIL	TNF-related apoptosis-inducing ligand
UGT1A*	UDP glucuronosyl transferase 1 family, polypeptides A*
UGT2B*	UDP glucuronosyl transferase 2 family, polypeptides B*
UKHTB	United Kingdom Human Tissue Bank
UW	University of Wisconsin Solution
vol	volume
V	Volts
WEM	William's Medium E

CHAPTER 1: Introduction

1.1 THE LIVER
- 1.1.1 Structure of the Liver
  - 1.1.1.1 Hepatocytes
  - 1.1.1.2 Cholangiocytes
  - 1.1.1.3 Kupffer Cells
- 1.1.2 Functions of the Liver
  - 1.1.2.1 Protein Synthesis
  - 1.1.2.2 Carbohydrate and Lipid Metabolism
  - 1.1.2.3 Drug Metabolism and Transport
- 1.1.3 Liver Function Tests

1.2 HEPATOTOXICITY
- 1.2.1 Types of Liver Damage Caused by Drugs
  - 1.2.1.1 Dose-dependent
  - 1.2.1.2 Idiosyncratic
- 1.2.2 Mechanisms of Liver Damage Caused by Drugs
  - 1.2.2.1 Apoptosis
  - 1.2.2.2 Oncosis
- 1.2.3 Risk Factors for Hepatotoxicity
  - 1.2.3.1 General Risk Factors
  - 1.2.3.2 Risk Factors for Hepatotoxicity in Patients with Cancer
- 1.2.4 Testing for Drug-Induced Hepatotoxicity
  - 1.2.4.1 Isolated enzymes, organelles and subcellular fractions
  - 1.2.4.2 Isolated cells *in vitro*
  - 1.2.4.3 Liver slices
  - 1.2.4.4 Isolated perfused liver
  - 1.2.4.5 Liver *in vivo*
  - 1.2.4.6 Clinical trial in humans
  - 1.2.4.7 Drug-Induced Hepatotoxicity in Oncology

1.3 HEPATOTOXICITY INDUCED BY ANTICANCER AGENTS
- 1.3.1 Chemotherapy-Induced Hepatotoxicity
  - 1.3.1.1 Type of chemotherapy (single-agents)
  - 1.3.1.2 Route of administration
- 1.3.2 Hepatotoxicity During Clinical Development of Novel Anticancer Agents

1.4 AIMS AND OBJECTIVES OF THIS STUDY

1.1 THE LIVER

The liver is the largest organ in the body, and it performs a wide range of physiologically essential functions. In order to understand the significance of damage to the liver, it is first necessary to consider the unique structure and functions of this organ. Common tests that are used to assess liver function and/or detect liver damage will also be considered.

1.1.1 Structure of the liver

Anatomically, the liver is divided into four lobes that are visible macroscopically: the left-, right-, quadrate- and caudate- lobes. The liver is functionally divided into eight segments based on the distribution of the portal and hepatic veins: each segment receives a branch of the portal vein and is an independent functional unit. At a microscopic level, the liver consists of many individual functional units, traditionally called lobules. Each lobule is bounded by four to five portal triads (supplied from the portal vein and hepatic artery) and has a central terminal hepatic venule (central vein) (Thomson and Shaffer, 2000). It is also possible to consider liver structure in terms of multiple acini, and this is considered to be a more physiologically sound concept than that of the liver lobule (Thomson and Shaffer, 2000). At the centre of the acinus is the portal triad (branches of the hepatic portal vein, hepatic artery and bile duct bound together), and the terminal hepatic venules are at the periphery. The acinus is divided into 3 zones based upon the distance from the blood vessels.

The acini are comprised of several cell types that include hepatocytes, cholangiocytes, Kupffer cells, stellate cells and Pit cells. Hepatocytes are arranged in interconnected sheets and cords that are one or two cells thick (see figure 1.1), with blood-filled spaces called sinusoids running between them (Thomson and Shaffer, 2000). Hepatocytes are in contact with the sinusoidal blood as it passes from the portal tracts to enter the terminal hepatic venules (Farrell, 1994). They are separated from the bloodstream by a single layer of sinusoidal endothelial cells with interspersed Kupffer cells (the liver's resident macrophages), stellate (fat-storing or Ito) cells and Pit cells (Thomson and Shaffer, 2000). The sinusoidal lining is fenestrated, allowing the exchange of molecules and small particles between

hepatocytes and the blood stream. The Kupffer cells form part of the reticuloendothelial system and phagocytose particulate matter in circulating blood (particularly spent erythrocytes), endotoxins and other noxious substances (Thomson and Shaffer, 2000). Stellate cells store vitamin A and produce extracellular matrix proteins such as collagens; they are also distributed among the sinusoidal endothelial cells like the Kupffer cells (Thomson and Shaffer, 2000). Pit cells are large, granular lymphocytes, which function as natural killer cells. The extracellular matrix of the liver includes its reticulin framework and several molecular forms of collagen, laminin, fibronectin and other extracellular glycoproteins.

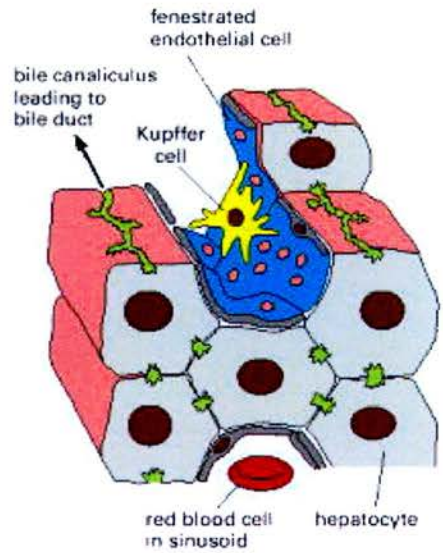


Figure 1.1: Schematic representation of the fine structure of the human liver
(Source: Alberts et al, 2002: Adaptation of figure 22-21)

Unlike most organs, which have a single blood supply, the liver receives blood from two sources. Oxygenated blood comes from the hepatic artery (25%), and oxygen-depleted blood comes from the intestinal tract via the hepatic portal vein (75%) (Thomson and Shaffer, 2000). Small branches of each blood vessel (the terminal portal venule and the terminal hepatic arteriole) enter the acinus at the portal triad (zone 1). Blood from the portal venules and hepatic arterioles then mixes and flows through the hepatic sinusoids between plates of hepatocytes (see figure 1.1), towards the terminal hepatic venule (zone 3) where blood from several acini merges (Farrell, 1994). This double vascularisation allows the absorption of substances from the

blood by sinusoids. The terminal hepatic venules then merge to form the hepatic vein, which carries the efferent blood to the inferior vena cava.

The hepatocytes also form a system of bile canaliculi into which they secrete waste products of their metabolism and bile, which is ultimately discharged into the gut via bile ducts (Thomson and Shaffer, 2000). The canaliculi are formed by grooves on the contact surface of adjacent hepatocytes. The biliary system forms another important feature of the liver. Secretion of bile salts, other organic anions and electrolytes occurs through the canalicular region of the plasma membrane of hepatocytes (see figure 1.1). Bile passes out of the canaliculi and into the bile ductules, which are composed of bile duct epithelial cells (cholangiocytes). Bile ductules pass to the portal and interlobular bile ducts, which then drain into the large intra-hepatic bile ducts, which eventually leave the liver via the porta hepatis (Thomson and Shaffer, 2000). There they unite to form the common hepatic duct. The cystic duct connects the gall bladder to the common hepatic duct, below which point the common bile duct descends to enter the duodenum (Farrell, 1994).

1.1.1.1 Hepatocytes

Hepatocytes are the predominant cell type in the liver and represent 60-80% of the number of cells, occupying approximately 80% of the liver volume (Bayliss & Skett, 1996). There are approximately 2.6×10^{11} hepatocytes in the normal adult liver (Yamashita et al, 2000), which are arranged in plates that radiate out from each portal triad toward adjacent central veins.

Hepatocytes are polarised cells which possess well defined basolateral (sinusoidal) and apical (canalicular) plasma membrane domains (Simons and Fuller, 1985). Membrane proteins are first transported to the basolateral surface and if necessary migrate to the apical side (Wessels et al, 1989). This is important since during *in vitro* culture basal proteins relocate to the apical side.

The nuclei in hepatocytes are round with dispersed chromatin and prominent nucleoli. The nuclei of hepatocytes vary in size more so than in many other cell

types, and reflects polyploidy. Over fifty percent of hepatocytes have a polyploid nucleus (Alberts et al., 2002). Binucleate cells are also common. Hepatocytes contain many mitochondria, and large amounts of rough endoplasmic reticulum and free ribosomes.

The liver is normally quiescent, and hepatocytes normally live for a year or more and are renewed at a slow rate (Alberts et al., 2002). Another capability of the liver, which is not common among other organs, is its ability to regenerate under specific conditions. After partial hepatectomy, infection, or exposure to chemical toxicants (alcohol, certain drugs), the liver has the capability to regenerate, whereby hepatocyte proliferation occurs. Problems may arise when regeneration of hepatocytes lost by apoptotic or oncotic cell death mask the detection of liver injury (Jaeschke et al, 2002). The liver is capable of rapidly increasing or decreasing its size by cell proliferation or cell death, when the need arises.

1.1.1.2 Cholangiocytes

Cholangiocytes are the epithelial cells that line the bile duct. They participate in bile formation and secretion, and are actively involved in the absorption and secretion of water, chloride, bile acids and glucose (Tietz and LaRusso, 2002). One enzyme that is present in cholangiocytes, and is used as a marker of liver function is gamma glautamyl transpeptidase (GGT). GGT activity in hepatocytes is prominent in fetal liver but disappears after birth. In cholangiocytes, GGT is involved in the synthesis of glutathione, playing a crucial role in maintaining both glutathione and cysteine levels: GGT breaks down glutathione to generate gamma-glutamyl compounds and cysteinylglycine, which is further cleaved by membrane-bound dipeptidases. The constituent amino acids are then taken up and used by cells for intracellular resynthesis of glutathione (Zhang et al, 2005).

1.1.1.3 Kupffer Cells

Kupffer cells are the macrophages of the liver, and form part of the reticuloendothelial system. Their primary function is to recycle old red blood cells that are no longer functional. However, they can also be involved in the liver's acute or chronic response to toxic compounds. Kupffer cells are able to transverse the

sinusoidal barrier and enter the space of Disse, facilitating their signalling function. Central to this signalling role is the ability of Kupffer cells to respond to local changes by the release of cytokines and other signalling molecules such as reactive oxygen species. Kupffer cell activation results in the release of an array of inflammatory mediators, growth factors and reactive oxygen species (Roberts et al, 2007). This activation appears to modulate acute hepatocyte injury as well as chronic liver responses. KC can be seen as the first responder to toxicants that release inflammatory mediators inducing subsequent hepatic damage. However, in some cases, cytokines released from KC appear to protect the liver from further damage. KC act also as antigen presenting cells and play in this respect an essential role in idiosyncratic liver toxicity (Uetrecht, 2007). Therefore Kupffer cells can act as either protector or as a mediator of damage, depending on the toxic agent.

1.1.2 Functions of the liver

The liver performs several functions, for which its intricate structure and variety of cell types is necessary. However, the majority of the functions of the liver are carried out by hepatocytes. The principal functions of the liver fall into two categories: functions of synthesis (proteins, carbohydrates, cholesterol, bile, lipids, urea); and functions of metabolism and excretion of endogenous and exogenous compounds (either via the biliary system or back into the blood stream). Hepatocytes are dependant on ATP for many functions, including ureagenesis, gluconeogenesis and fatty acid metabolism among multiple other metabolic processes (Jaeschke et al, 2002).

1.1.2.1 Protein Synthesis

One of the main roles of hepatocytes is the synthesis and secretion of the majority of proteins that circulate in the plasma: albumin, and most of the globulins other than gamma globulins. Globulins include the clotting factors: fibrinogen, prothrombin (factor II), and factors V, VII, IX and X. They also produce several structural proteins and enzymes. As one of the functions of the liver is to synthesise albumin, a change in the synthetic functions of the liver can alter the disposition of drugs that are tightly bound to albumin (DeLeve, 2003). Albumin and clotting factors can be

used as markers of liver injury. However, the half-life of albumin is approximately 20 days and its detection is more useful in chronic rather than acute liver disease. Because of its abundance, a decrease in albumin levels corresponds to a reduction in liver capacity.

1.1.2.2 Carbohydrate and Lipid Metabolism

The liver also plays a central role in energy status as the major store of glycogen and as the site of gluconeogenesis (forming glucose from non-carbohydrate sources such as amino acids) (Bayliss & Skett, 1996). The liver forms fatty acids from carbohydrates and synthesises triglycerides from fatty acids and glycerol. The liver packages triglycerides with cholesterol, phospholipids and an apoprotein into a lipoprotein. The lipoprotein enters blood for utilisation or storage in adipocytes. Most cholesterol synthesis also takes place in the liver, and bile salts are the major product of cholesterol catabolism. The liver is the sole site of bile salt formation.

A large number of enzymes are involved various pathways of metabolism in the liver, but two are often used to assess liver function or liver damage: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST). AST is present in various tissues while ALT is predominantly expressed in liver and kidneys.

1.1.2.3 Drug Metabolism and Transport

Xenobiotic metabolism and transport (uptake and excretion) is an important function of the liver. Several steps are involved in drug handling by the liver: uptake from the blood by diffusion or by carrier-mediated transport at the sinusoidal pole of the hepatocyte, metabolism by phase I and phase II reactions within the hepatocyte; active transport across the canalicular pole into bile; and excretion via the bile into the intestine (DeLeve, 2003). Examples of drug metabolising enzymes involved in anti-cancer drug metabolism are provided in table 1.1. The liver is the principal organ involved in drug metabolism; other sites of drug metabolism include epithelial cells of the gastrointestinal tract, lungs, kidneys and the skin. The metabolic functions of the liver are performed by the hepatocytes and therefore transport of drugs and their metabolites into and out of hepatocytes is also important.

Drugs administered orally which are then absorbed from the stomach and the intestine must pass through the liver before being transported throughout the body (and to its target) by the circulation. These compounds are subjected to 'first pass' metabolism in the liver (and the small intestine where some CYP enzymes are present). A drug that escapes first pass metabolism maintains its biological activity, and other routes of administration that avoid first-pass metabolism include intravenous and intramuscular.

It should be noted that while drug metabolism is required to efficiently eliminate these compounds from the body, certain chemicals are metabolically activated to reactive derivatives that cause cell toxicity and cancer. Among these, the CYP450s that metabolically activate toxicants and carcinogens are limited to a few forms including CYP1A1, CYP1A2, CYP1B1, CYP2A6, and CYP2E1. CYP1A1 and CYP1B1 metabolically activate polycyclic aromatic hydrocarbons such as benzo[a]pyrene. CYP1A2 carries out the N-oxidation of arylamines and heterocyclic amine food mutagens and CYP2E1 metabolises numerous low-molecular-weight toxicants and suspected carcinogenic agents (Gonzalez, 2005).

Table 1.1: Drug metabolising enzymes and examples of their anti-cancer drug substrates (adapted from Harmsen et al, 2007)

Phase	Drug metabolising enzyme	Substrates
Phase I	CYP1A1	Dacarbazine, docetaxel, erlotinib, tamoxifen
	CYP1A2	Dacarbazine, erlotinib, etoposide, flutamide, imatinib, tamoxifen
	CYP2A6	Cyclophosphamide, ifosfamide, letrozole, tegafur
	CYP2B6	Cyclophosphamide ifosfamide, tamoxifen
	CYP2C8	Cyclophosphamide, docetaxel, ifosfamide, paclitaxel, tegafur, tretinoin
	CYP2C9	Cyclophosphamide, ifosfamide, imatinib, tamoxifen, tegafur, tretinoin
	CYP2C19	Cyclophosphamide, ifosfamide, imatinib, tamoxifen, thalidomide
	CYP2D6	Imatinib, tamoxifen, vinorelbine
	CYP2E1	Cisplatin, etoposide, tamoxifen, tretinoin, vinorelbine
	CYP3A4/5	Busulfan, cisplatin, cyclophosphamide, cytarabine, docetaxel, doxorubicin, erlotonib, etoposide, flutamide, fulvestrant, gefitinib, ifosfamide, imatinib, irinotecan, letrozole, mitoxantrone, paclitaxel, tamoxifen, teniposide, topotecan, tretinoin, vinblastine, vincristine, vindesine, vinorelbine
Phase II	UGT*	Doxorubicin, epirubicin, etoposide, irinotecan, topotecan, tamoxifen
	SULT*	Tamoxifen
	GST*	Busulfan, chlorambucil, cyclophosphamide, doxorubicin, ifosfamide, melphalan, nitrosurea

1.1.2.3.1 Phase I Reactions

Phase I drug metabolism includes oxidation, reduction, hydrolysis and hydration reactions (Gibson and Skett, 2001). In most cases, the final product of these reactions contains a chemically reactive functional group and is prepared for phase II (conjugation) reactions. If the metabolites of phase I reactions are sufficiently polar, they may be readily excreted following phase I metabolism. Phase I enzymes include Cytochrome P450s, flavin-containing mono-oxygenases, and epoxide hydrolases. Oxidation reactions performed by the microsomal mixed-function oxidase system (cytochrome P450-dependent) are arguably the most studied family

of drug metabolising enzymes, and this enzyme system carries out a wide diversity of reactions and has a wide variety of substrates.

The most widely studied drug-metabolising enzymes are the cytochrome P450-dependent mixed-function oxidases. Cloning and sequencing of CYP450 cDNAs and, more recently, total genome sequencing have revealed the existence of 57 putatively functional genes and 58 pseudogenes in humans (Nelson, 2006). These genes are grouped, based on amino acid sequence similarity, into a large number of families and are found embedded in the membrane of the endoplasmic reticulum. CYP P450s are principally expressed in hepatocytes. However, some P450s are also expressed at lower levels in gut, lung, kidney and haematopoietic tissue (Gonzalez, 2005). CYP450s are complex and diverse in their regulation and catalytic activities.

1.1.2.3.1.1 Cytochrome P450s activity

A limited number of CYP450s (15 in humans) are primarily involved in xenobiotic metabolism: CYP450 families 1 to 3. Oxidation reactions involve the addition of oxygen or removal of a hydrogen, catalysed by mixed function oxidases and mono-oxygenases in the liver. These oxidative reactions typically involve a CYP450 haemoprotein, NADPH and oxygen. CYP450 can also catalyse reduction and peroxidation reactions.

The central features of the cytochrome P450 catalytic cycle are the ability of the haem iron to undergo cyclic oxidation/reduction in conjunction with substrate (drug) binding and oxygen activation, as shown in figure 1.2. Step 1 involves drug binding to the oxidised (ferric Fe3+) form of CYP450. Step 2 involves the first electron reduction of substrate-bound ferric CYP450 to the ferrous (Fe2+) form of the haemoprotein, and requires NADPH H+ and NADPH-CYP450 reductase. Step 3 involves binding of molecular oxygen to the binary ferrous CYP450-substrate complex. Steps 4, 5, and 6 involve putative electron rearrangement, introduction of the second and subsequent oxygen insertion into the drug substrate and hydoxylated metabolite release. Step 5 involves NADPH CYP450 reductase or cytochrome b_5 and its associated flavoprotein reductase.

CYP3A4 is the most abundant P450 in the liver and plays a role in the metabolism of approximately half of all the drugs on the market and in development. CYP3A4 exhibits considerable inter-individual variation in expression in the human population by one to two orders of magnitude (Gibson and Skett, 2001). The CYP3A family metabolises many drugs, and several anticancer agents including docetaxel, etoposide, gefitinib, imatinib, irinotecan, tamoxifen, vinblastine and vinristine (Rodriguez-Antona and Ingelman-Sundberg, 2006).

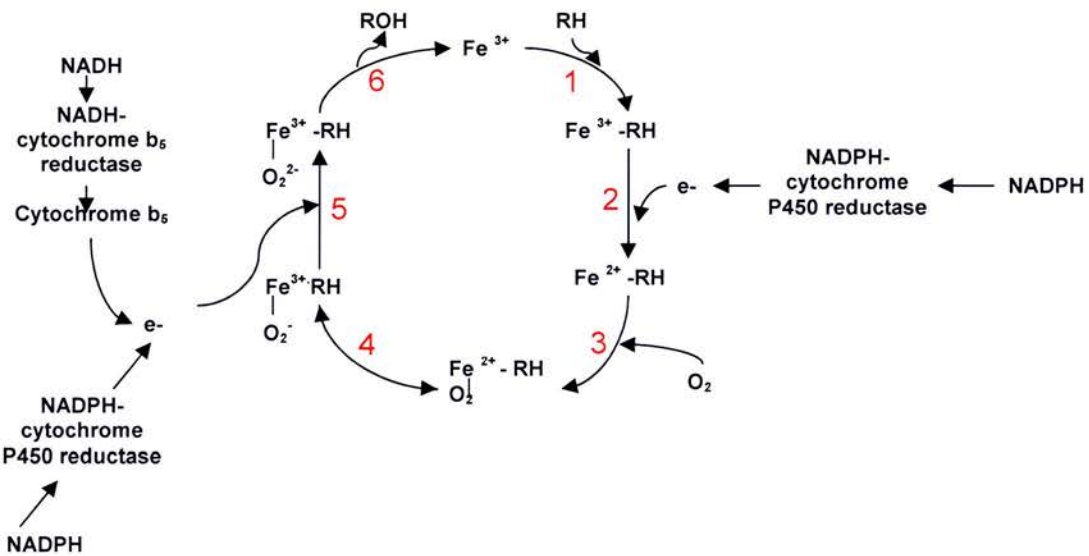


Figure 1.2: Catalytic cycle of Cytochrome P450

RH represents the drug substrate and ROH the corresponding hydroxylated metabolite.

1.1.2.3.1.2 Cytochrome P450 Regulation

The metabolism of drugs is substantially influenced by the passive or deliberate intake of many chemical substances. Exposure to these substances may be either in the environment, for medical reasons or as a result of lifestyle. These chemical substances come from a variety of sources including pharmaceutical products, cosmetics, food additives and industrial chemicals.

The duration and intensity of the pharmacological action of many drugs is influenced by their rate of metabolism. Therefore inducers and inhibitors of drug metabolism play a key role in the pharmacological effect of many drugs, as they can alter the expression and activity of many drug metabolising enzymes. Inducers of drug metabolism have the potential to decrease the duration and intensity of drug action, whereas the opposite is true for inhibitors. Consideration is given here to the CYP450 families of enzymes, however these principles of gene induction and inhibition hold true for several of the other drug metabolising enzymes. Many drugs are known to induce either their own metabolism or the biotransformation of other drugs. The induction of specific liver enzymes, particularly the CYP450s, plays a role and has implications in clinical pharmacology and toxicology. One example of this is the antibiotic rifampicin, which is a CYP3A4 inducer. Rifampicin-induction of CYP3A4 can cause increased clearance of co-administered drugs such as warfarin, and verapamil.

The mechanisms underlying induction of drug metabolising enzymes, and particularly that of the CYP450s have been extensively studied. Induction of drug metabolism may arise as a consequence of increased synthesis, decreased degradation, activation of pre-existing components or a combination of the three. However, the majority of CYP450s are transcriptionally regulated, and induction involves ligand-dependent nuclear receptors (NR) (Nakata et al, 2006).

The mechanism of induction of many of the CYP450s that are involved in drug metabolism has been investigated and include CYP1A1, and CYP2B*, CYP3A*. CYP1A1 is of interest because of its ability to activate environmental compounds, such as polyaromatic hydrocarbons (PAH), to biologically reactive metabolites that interact with DNA, resulting in chemical carcinogenesis. CYP1A1 is present in low amounts in non-induced liver (approximately 2-5% of the total CYP450 content) but increases approx 8-16 fold upon induction. CYP1A1 is induced by a wide variety of environmental chemicals including PAHs (e.g. benzo[a]pyrene, β-naphthoflavone), and is associated with a specific cytosolic receptor; the aryl hydrocarbon receptor (AhR) (Whitlock, 1999). As shown in figure 1.3A, PAHs enter the cell and combine

with cytosolic AhR. Inducer-free Ahr exists in combination with at least two other proteins (heat shock protein 90 [Hsp90] and AhR-interacting protein [AIP]), the function of which is not entirely clear but they are though to maintain the AhR in a receptive configuration for induced binding. The inducer-receptor complex translocates to the nucleus where it heterodimerises with the AhR nuclear translocator protein (Arnt). This complex (probably in conjunction with other transcription factors) targets several genes in the nucleus including CYP1A1. An initiation complex forms at the CYP1A1 promoter and increases the rate of mRNA synthesis. Large amounts of newly translated CYP1A1 protein are then incorporated into the membrane of the endomplasmic reticulum (along with haem insertion) resulting in CYP1A1 induction (Whitlock, 1999). The AhR/Arnt system is not solely dedicated to transcriptional activation of CYP1A1, but is involved in the activation of several other genes including other xenobiotic metabolising enzymes.

CYP3A4 is the predominantly expressed CYP isoform in the liver, and is critical in the metabolism of many clinically used drugs: it is estimated that CYP3A4 is responsible for approximately 60% of the cytochrome P450-mediated metabolism of pharmaceuticals in therapeutic use, in addition to metabolising a number of endogenous compounds (e.g. testosterone) and environmental xenobiotics (e.g. aflotoxins) (Gibson and Skett, 2000). This isoform is highly inducible in man by synthetic/natural steroids (e.g. dexamethasone), and drugs such as rifampicin and phenobarbitone. Regulation of CYP3A* genes (CYP3A4, CYP3A5, CYP3A7) is complex, mainly due to the number of regulatory elements found in both the distal enhancer and the proximal promoter. Similar to CYP1A1 induction, cellular receptors play a critical role in regulation of CYP3A* gene expression, the most important of which is pregnane X receptor (PXR) (Pascussi et al 2001). Several cellular receptors are involved in CYP3A* induction (figure 1.3B), and inducers may show preferential specificity for one receptor or interact with more than one receptor. PXR is a member of the nuclear receptor family and plays an important role in CYP3A4 gene regulation for several xenobiotic and endogenous steroid inducers. PXR possesses a ligand-(inducer)-binding domain and a DNA binding domain. PXR/RXR heterodimer formation is essential for the activation of CYP3A4 reporter

gene constructs by xenobiotics and naturally occurring pregnane steroids. In addition, two further receptors mediate induction of the CYP3As, namely the glucocorticoid receptor and the constitutive androstane receptor (CAR), the latter also forming a heterodimer complex with the RXR (Pascussi et al 2001). CAR has been shown to bind inducers, followed by binding of the inducer-receptor complex to the corresponding response element in CYP3A* promoters, however the precise role of the GR is unknown.

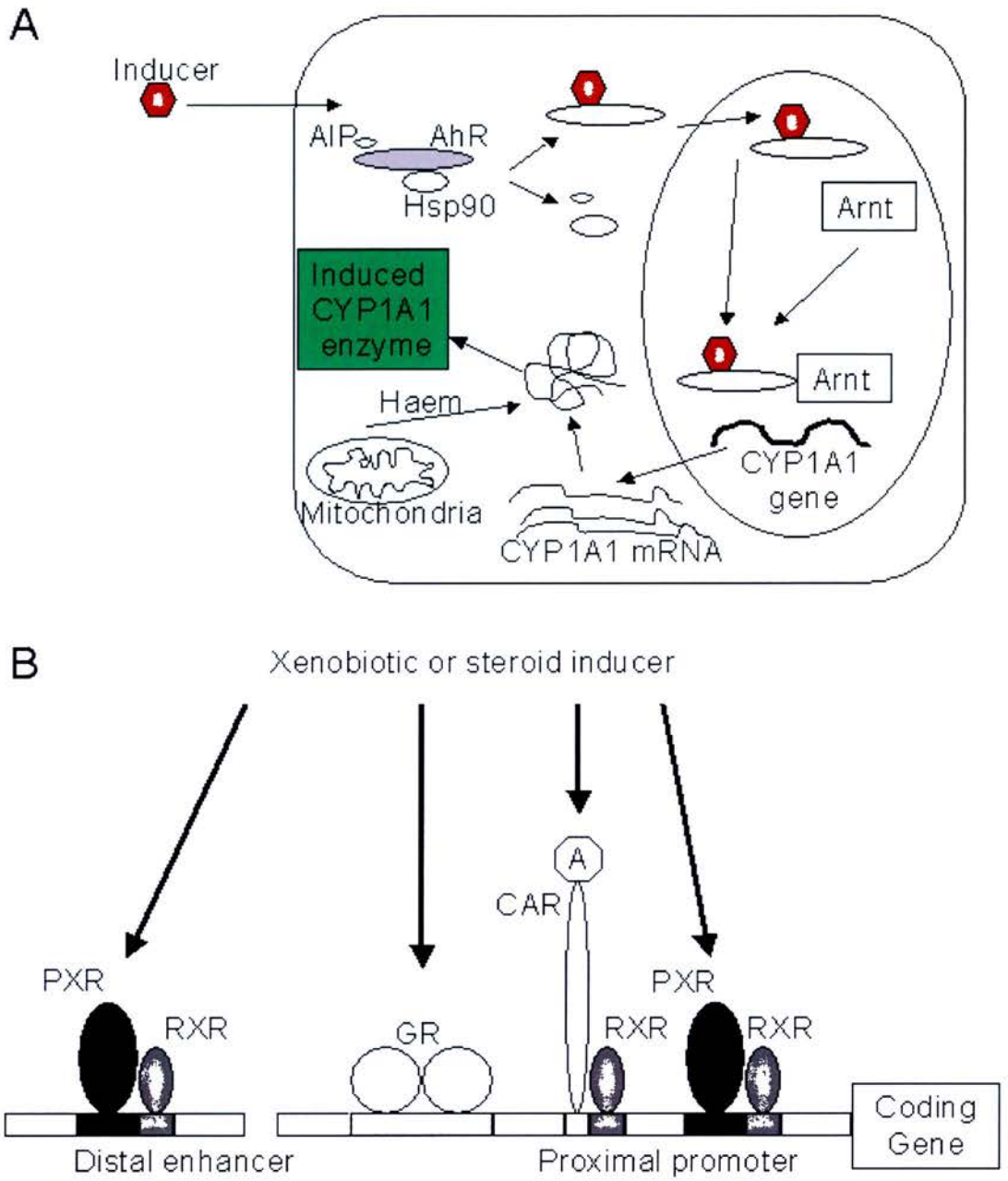


Figure 1.3: Receptor-dependent CYP induction in hepatocytes

A) CYP1A1 induction: Polyaromatic hydrocarbons (PAHs) act as a CYP1A1-inducer, binding to Aryl hydrocarbon receptor [AhR] (that was previously bound in cytosol to Ahr-interacting protein [AIP] and heat shock protein 90 [Hso90]). The inducer-AhR complex translocates to the nucleus where it heterodimerises with AhR nuclear translocator (Arnt) and targets the CYP1A1 gene. It binds at the promoter, increases mRNA synthesis and results in induced CYP1A1. B) CYP3A4 induction: In the nucleus of hepatocytes, xenobiotics or steroid inducers bind to nuclear receptors (pregnane X receptor [PXR], retinoid X receptor [RXR], glucocorticoid receptor [GR], constitutive androstane receptor [CAR] and inducer-receptor complexes bind response elements in CYP3A4 promoter, increasing mRNA synthesis and results in induced CYP3A4. (Adapted from Gibson and Skett, 2001)

These two examples demonstrate CYP450 induction in hepatocytes, however similar nuclear-receptor mediated induction occurs for several drug-metabolising enzymes (phase I and phase II) and drug transporters. The different NRs along with their target genes are shown in table 1.2. Despite the large numbers of ligands that can interact with each nuclear receptor, the sets of genes activated by these ligands are very different. With the example of PXR, this is due to a change in conformation after ligand binding (Poso and Honkakoski, 2006). PXR and CAR have one of the broadest spectrums of gene regulation as they regulate note only CYPs but also other proteins such as drug transporters (Timsit and Negishi, 2007).

Table 1.2: Regulation of drug metabolising enzymes and transporters

Ligand	Nuclear Receptor	Phase I	Phase II	Phase III
xenobiotics	Ahr	CYP1A1 CYP1A2 CYP1B1	UGT1A1 UGT1A6	ABCG2 (BCRP)
xenobiotics	CAR	CYP2A6 CYP2B1 CYP2B6 CYP2C9 CYP2C19	UGT1A1	ABCC2 (MRP2) ABCC3 (MRP3) ABCC4 (MRP4)
xenobiotics	PXR	CYP1A2 CYP2B6 CYP2C9 CYP2C19 CYP3A4 CYP3A7 CYP7A1 CYP3A	SULT2A1 UGT1A1 UGT1A3 UGT1A4	ABCA1 ABCB1 (MDR1) ABCB11 (BSEP) ABCC1 (MRP1) ABCC2 (MRP2) ABCC3 (MRP3) ABCG2 (BCRP)

Conversely to the induction of drug metabolising enzymes discussed above, inhibition can also occur. Several cases of inhibition of CYP450 enzymes have been well defined. Many xenobiotics of diverse chemical structure can act through a variety of mechanisms to decrease the biotransformation of drugs. The inhibition of drug metabolism by xenobiotics can take place in several ways, including the destruction of pre-existing enzymes, inhibition of enzyme synthesis or by binding to and thus inactivating the drug metabolising enzyme. Inhibition of a particular drug

metabolising enzyme can occur when there are co-substrates; two (or more) drugs can compete for the same drug metabolising enzyme and dependent on their relative affinities and inhibitory potencies can result in inhibition of metabolism of one of the drugs, which leads to accumulation of one substance, and can induce toxic effects (drug-drug interaction). One example of this phenomenon is in the inhibition of CYP3A4 by clotrimazole and saquinavir. Another cause of inhibition of drug metabolism is destruction of functional CYP450s in the liver. Xenobiotics containing an olefinic (C=C) or acetylinic (C≡C) function are activated by CYP450s, resulting in metabolites that cause CYP450 haem destruction. Examples of compounds that undergo suicidal activation by CYP450s include the olefinic compounds ethylene and vinyl chloride, and the acetylenic compound acetylene. Metal ions are also capable of inhibiting functional oxidase activity by a variety of mechanisms (dependant on the metal in question). One example of this is the metal ion cobalt that exerts its inhibitory effect on drug metabolism by modulating the synthesis and degradation of the haem prosthetic group of CYP450. In addition to modulating the synthesis and/or degradation of CYP450s, some xenobiotics can inhibit drug metabolism by forming inactive complexes with CYP450s. These compounds are substrates of CYP450 and require metabolism to exert their full inhibitory effect. Inhibitors forming complexes are metabolised by CYP450, forming a metabolic product that binds tightly to the haemoprotein that originally formed it, thus preventing its further participation in drug metabolism. Examples of drugs which can act in this way include amphetamine, cimetidine, isoniazid and methadone.

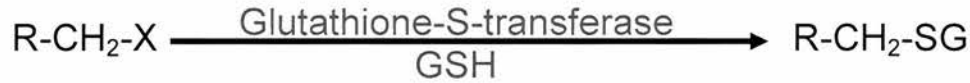
1.1.2.3.2 Phase II Reactions

Many products of phase I drug metabolism are not eliminated rapidly and undergo a phase II reaction, generally considered to be detoxification reactions. These reactions involve an endogenous substrate combining with the newly incorporated functional group on the drug molecule to form a highly polar conjugate. The result is therefore a water-soluble metabolite that can be excreted in urine or bile. Phase II reactions are also known as conjugation reactions and enzymes that carry out these reactions include UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT),

Glutathione S-transferases (GSTs), and N-acetyltransferases. The phase II reactions are usually faster than phase I reactions and therefore the rate-limiting step in the metabolism of a compound is usually the phase I reaction.

Glucuronidation is quantitively the most important of all the conjugation reactions, and involves conjugation of drugs and endogenous compounds to glucuronic acid. Many functional groups (hydroxyl, carboxyl, amino, sulfhydryl) present in phase-I metabolised drug molecules have the potential to be glucuronidated, and certain drugs can be directly conjugated with glucuronic acid without the need for phase I metabolism. The enzyme that catalyses the conjugation of drugs with glucuronic acid is UDP_glucuronosyl transferase (UGT). UGT exists as a family of structurally related, although catalytically distinct, multiple forms and approximately 16 have been detected in man (Gibson and Skett, 2001). UGTs 1A1, 1A3, 1A4, 1A6, 2B4, 2B7, 2B10, 2B11 and 2B15 are all expressed in the human liver. The isoforms are divided into families based on their amino acid sequence similarity, similar to the CYP450s. The resulting drug-glucuronide conjugate is either excreted in the urine or faeces and the molecular weight of the conjugate is a critical determinant of the route of excretion. For example glucuronide conjugates with molecular weight greater than 400 daltons (i.e. drugs with molecular weight greater than 200 daltons) are excreted predominantly in the bile primarily, whereas lower molecular weight conjugates primarily undergo urinary excretion. Therefore high molecular weight drugs such as morphine and chloramphenicol are excreted in the bile and hence into the intestine. The intestinal flora contains significant amounts of the enzyme β-glucuronidase, an enzyme that catalyses the hydrolysis of the glucuronide conjugate, resulting in the re-formation of free drug (parent compound or phase I metabolite), which can then be reabsorbed from the intestine, transported to the liver and then undergoes re-conjugation and re-excretion. This loop is called the entero-hepatic circulation and may make a contribution to prolonging the half-life of the drug in the body, potentiating the pharmacological action of the drug, providing that sufficiently high blood levels of the drug are achieved. This would show in a pharmacokinetic profile as a return of free metabolite and has been reported for the anti-cancer topoisomerase inhibitor irinotecan and its metabolite SN-38 (Canal et al, 1996).

The glutathione-S-transferase (GST) family of enzymes are widely distributed through the body, particularly in the cytosol of hepatocytes. They catalyse the conjugation of a variety of compounds with the endogenous tripeptide (Gly-Cys-Glu) glutathione (glutamylcysteinylglycine, GSH), as follows:



where $R\text{-}CH_2\text{-}X$ represents hundreds of electrophilic drugs and xenobiotics, and $R\text{-}CH_2\text{-}SG$ represents the drug-glutathione adduct. Glutathione is recognised as a protective compound within the body for the removal of potentially toxic electrophilic compounds. Many drugs are, or are metabolised by phase I metabolism to, strong electrophiles, and these can generally be conjugated to glutathione to form (usually) non-toxic conjugates. Glutathione conjugates can be excreted in urine, or more usually in bile, but more often further metabolism of the conjugates takes place. Once glutathione is conjugated to the drug, it can be attacked by γ-glutamyltranspeptidase, which removes the glutamate, and a peptidase which removes the glycine, to yield the cysteine conjugate. N-acetylation of the cysteine pathway can then occur to yield the N-acetylcysteine conjugate or mercapturic acid. The glycyl-cysteine and cysteine conjugates and the mercapturic acids are all found as excretion products depending on the substrate and species under study. Substrates for GSTs include epoxides, alkanes, alkenes, and aromatic halo- and nitro-compounds. In addition to their ability to catalyse the above conjugation reaction (e.g. paracetamol), GSTs have the ability to bind a variety of endogenous and exogenous substrates without metabolism (e.g. oestradiol, tetracycline, penicillin).

1.1.2.3.3 Drug uptake and excretion

Membrane transporters are present in several organs throughout the body, and play a vital role in the liver where they are involved in the uptake and excretion of drugs, and can influence their safety profile. Membrane transporters play such an important role in the liver in the disposition of drugs that they are sometimes referred to as phase III detoxification systems (Nakata et al, 2006). In the liver, membrane transporters are primarily expressed in hepatocytes and cholangiocytes.

Lipophilic molecules may move from plasma to hepatic cytosol by simple or facilitated diffusion (Chandra and Brouwer, 2004). However, numerous transport proteins are present in the basolateral plasma membrane of hepatocytes to mediate uptake of amphipathic and polar organic compounds, as well as some lipophilic molecules, from sinusoidal plasma to hepatic cytosol (Chandra and Brouwer, 2004). Transport proteins in hepatocytes also play an important role in the excretion of drugs and metabolites, with uni- or bi-directional basolateral transport systems transporting polar molecules from hepatic cytosol in to the sinusoidal blood. Active transport proteins in the canalicular plasma membrane of hepatocytes are responsible for the biliary excretion of drugs and metabolites.

The sinusoidal, basolateral transport proteins, belong to the gene superfamily of solute carriers, and mediate the movement of compounds to and from the sinusoidal blood. They comprise the organic anion transporting polypeptides (OATPs), Na^+-taurochlorate co-transporting polypeptide (NTCP), multi-drug resistance-associated proteins (MRPs), and organic anion and cation transporters (OATs and OCTs) (Chandra and Brouwer, 2004). OATPs play an important role in the hepatic clearance of many drugs, transporting a large variety of organic cations as well as some cations, and neutral steroids. Within this family OATP1A2, 1B1, 1B3 and 2B1 are the proteins predominantly expressed in the human liver, and these are sodium independent and bi-directional. The Na+ -taurochlorate co-transporting polypeptide (NTCP) is a sodium dependent transporter responsible for bile salt uptake. OATs are organic anion transporters, and OAT2, OAT4 and OAT5 are expressed in the human liver. The MRPs are ATP-dependent, and belong to the ATP binding cassette (ABC) family of transporters. They transport organic anions (e.g. drugs conjugated to glutathione, glucuronate or sulphate) out of hepatocytes (Borst et al, 2000).

Bile is an important route for the elimination of drugs, particularly those that have been biotransformed by the liver (Farrell, 1994). The biliary excretion of xenobiotics and metabolites occurs predominantly by unidirectional ATP-dependent export pumps that transport substrates across the canalicular membrane of hepatocytes into

bile (Chandra and Brouwer, 2004). These canalicular transport proteins belong to the ABC superfamily of transporters (Hyde et al, 1990). MDR1 (ABCB1) is the most extensively studied canalicular transporter and it produces p-glycoprotein. It primarily transports hydrophobic cations, and typical MDR1 substrates include many anticancer agents (see table 1.3) and several other xenobiotics (e.g. digoxin, methadone, cyclosporin A) (Ambudkar et al, 1999). MDR1 expression is highly regulated by the nuclear receptor PXR (Geick et al, 2001; Nakata et al, 2006). Bile salt export pump (BSEP) transports bile salts. Although not playing a major role in drug excretion, if its action is inhibited by a drug (e.g. Bosentan) intracellular bile salts can accumulate which can lead to hepatotoxicity (Fattinger et al, 2001). MRP2 excretes divalent bile salts, and glutathione- glucuronide- and sulphate- conjugates into bile (Konig et al, 1999). Breast cancer resistance protein (BCRP) may play an important role in the biliary excretion of sulphated conjugates of steroids and xenobiotics, and can confer resistance to anti-cancer compounds such as mitoxantrone and daunorubicin (Chandra and Brouwer, 2004).

Table 1.3 presents commonly identified anti-cancer drug substrates for the human transport proteins, which are present in the hepatic basolateral and canalicular membranes.

Table 1.3: Drug transporters and examples of their anti-cancer drug substrates (adapted from Harmsen et al, 2007 and Ambudkar et al, 1999)

Location	Drug transporter	Substrates
Canalicular	MDR1 (P-gp)	Daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, idarubicin, methotrexate, mithramycin, mitoxantrone, paclitaxel, teniposide, vinblastine, vincristine
	MRP2	Camptothecin, cisplatin, irinotecan, doxorubicin, etoposide, methotrexate, SN-38, vinblastine, vincristine
	BCRP	Imatinib, methotrexate, mitoxantrone, SN-38, topotecan
Sinusoidal	MRP1	Arsenic trioxide, chlorambucil, daunorubicin, doxorubicin, epirubicin, etoposide, melphalan, methotrexate, mitoxantrone, paclitaxel, vinblastine, vincristine
	MRP3	Carboplatin, cisplatin, doxorubicin, epirubicin, etoposide, methotrexate, teniposide, vinblastine, vincristine
	MRP4	Cyclic nucleotide anologues, methotrexate
	MRP5	Doxorubicin, methotrexate, nucleotide anologues, topotecan
	MRP6	Doxorubicin, etoposide, teniposide
	MRP8	5-Fluorouracil and its metabolites

1.1.2.3.4 Factors affecting drug metabolism and excretion

There are several factors that can affect drug metabolism in an individual. These include: inherent factors such as species and genetic differences, age, sex, hormones and diseases; and external factors such as dietary and environmental factors, and can alter the host's drug response. The contribution of these components may differ between different drugs and may also influence the susceptibility of an individual to drug-induced hepatotoxicity (Ingelman-Sundberg, 2001).

There are species differences in drug metabolising enzymes and transporters between humans and rodents. This can cause problems when a drug is tested pre-clinically in animal models and is then administered to humans. One example is the formation of toxic metabolites that are produced in humans but not in animals due to lack of

metabolic pathway. The genes regulated by PXR differ between mouse, rat and human, making it difficult to predict interactions in humans based on data from rodents (Hartley et al, 2004).

Genetic variations and polymorphisms between human individuals can also affect drug metabolism, and accounts for some of the variability in the effect of drugs. Members of the CYP450s enzyme family are highly polymorphic such as CYP2C19 and CYP2D6, and can influence drug disposition. Historically, drugs were tested in a relatively small population of patients and following approval of a drug, a much larger population is exposed to the drug and the effects of rare polymorphisms may then appear. For example CYP2C19 and CYP2D6 are highly polymorphic, which can result in 'ultrarapid' and 'poor' metabolisers, and the anti-cancer agents that they metabolise can be found in table 1.1. For CYP2C19 at least 15 variant alleles have been discovered of which seven encode no enzyme activity, and for CYP2D6 over 40 variant alleles have been described (van Schaik, 2005). Several CYP3A4 single nucleotide polymorphisms have been reported, and 18 alleles encoding amino acid changes are known (van Schaik, 2005). The impact of these polymorphisms on CYP activity is yet to be established. Phase II enzymes also have genetic polymorphisms influencing their activity. UGT1A1 is polymorphic which can influence the toxicity of the irinotecan (Iyer et al, 1998). The MDR1 gene is polymorphic and can impact in some cases on the disposition of drugs that are MDR1 substrates, and may have consequences for treatment efficacy (Marzolini et al, 2004).

External influences including dietary and environmental factors also have the potential to impact on drug metabolism. Dietary factors including protein, fat, carbohydrate, vitamin and alcohol intake as well as intake of tobacco smoke can influence drug metabolism. Whilst it is of interest that environmental exposure to petroleum products, heavy metals, pesticides and industrial pollutants can influence drug metabolism, little can be done to control these.

1.1.3 Liver Function Tests

To determine the function of, and damage to, the liver in humans, a series of biochemical markers may be measured. In a blood sample from the patient, levels of serum alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), bilirubin, albumin and clotting factors may be measured. Prothrombin time may also be measured. Despite their name, liver function tests (LFTs) do not directly determine the function of the liver. These markers can be spilt into two groups: those that determine function; and those that indicate toxicity. Serum albumin, bilirubin and prothrombin time are true LFTs for assessment of hepatic function, whereas enzymes (AST, ALT, ALP and GGT) are markers allowing detection of liver injury.

There are commonly accepted 'normal' limits for levels of each of these markers in blood, and the defined normal limits for LFTs can vary depending on factors such as body mass index, race, age and sex. For example, for the aminotransferases AST and ALT upper reference limits do not change for individuals aged 25 to 60, however those aged <25 or >60 will require age-adjusted reference limits when determing acceptable levels of AST and ALT (Dufour et al, 2000). However a guideline of normal limits can be seen in table 1.4. Changes in these in the same individual over a period of time, or levels above the upper reference limits may indicate that the liver is not functioning as normal. LFTs may be abnormal for a variety of reasons, and could indicate the presence of many kinds of liver disease or a toxic compound. A wide variety of mechanisms could result in abnormal LFTs, and include decreased hepatocyte function, hepatocyte transport defects, cell membrane damage or disruption of bile flow.

Table 1.4: Normal values of LFTs

(Source: Limdi and Hyde, 2003). IU= international units

Liver Tests	Normal limits
ALP	30-120 IU/L
Bilirubin	2-17 μmol/L
GGT	0-30 IU/L
Albumin	40-60 g/L
AST	0-35 IU/L
ALT	0-45 IU/L
Prothrombin time	10.9-12.5 seconds

Whilst these tests are routinely used, it should be noted that they provide clues as to the condition of the liver but do not always allow diagnosis of particular liver disease states. In certain cases there may be a disconnect between enzyme levels and function of the liver for example in cirrhosis where there is ongoing damage to the liver but patients will normally have normal or only slightly elevated AST and ALT. In this case AST and ALT lack sensitivity in detecting a chronic liver disease. Also worthy of mention is the fact that not all of the LFTs determined will be altered immediately following liver damage. For example, the half-life of albumin in plasma is approximately 20 days and therefore serum albumin levels change slowly in response to alterations in albumin synthesis (Johnston, 1999).

AST is expressed in several tissues throughout the body while ALT is primarily expressed in the liver and kidney. Changes in ALT levels are therefore more specific for liver damage that AST, and liver injury generally induces a greater increase in ALT than AST. The half-life of both AST and ALT are short: 17±5 and 47±10 hours respectively (Dufour et al, 2000). ALP is a membrane-bound enzyme markedly expressed at the canalicular pole of hepatocytes and cholangiocytes and is secreted in bile (Alvaro et al, 2000). It is involved in the transport of metabolites across cell membranes and is found in several other tissues at higher levels than in the liver. The half life of the liver isoenzyme is approximately 3 days (Dufour et al, 2000). Its increase in plasma during liver injury is due to retrograde reflux of biliary ALP,

enhanced hepatic synthesis and release into the serum, and induction of the intestinal AP form (Poelstra, 1997). Cholestasis stimulates synthesis of ALP by hepatocytes, and bile salts facilitate release of ALP from cell membranes into the plasma (Moss, 1997). In patients with cholestatic liver disease it is likely that jaundice may be present.

GGT is another membrane-bound enzyme that is present in the liver and has a similar localisation to ALP. GGT activity in serum comes primarily from the liver and the half-life of GGT is approximately 7 to 10 days (increases up to 28 days in alcohol-related liver disease) (Dufour et al, 2000). Bilirubin is rapidly conjugated by UGT and conjugated bilirubin is excreted in the bile, therefore being essentially absent from the blood. It is rapidly bound to albumin to form δ-bilirubin. Presence of δ-bilirubin corresponds to jaundice, and its half life is approximately equal to that of albumin. Total protein and albumin are not very informative during acute liver injury as they have relatively long half-lives. Similarly, prothrombin time is relatively insensitive as it does not increase unless each clotting factor is <10% of normal.

Prior to treatment with anti-cancer cytotoxic drugs, patients require careful assessment of liver function (King and Perry, 2001). LFTs are routinely performed prior to the onset of every cycle of chemotherapy. LFTs performed before treatment begins allow baseline readings for each patient and indication of any pre-existing liver disease. Furthermore, these tests are routinely performed before every cycle of chemotherapy and are used to detect hepatotoxicity in patients. Chemotherapeutic agents commonly cause transient LFT abnormalities with elevations in liver enzymes, without clinical evidence of liver impairment. Mild or moderate transient elevations of LFTs without clinical toxicity may often be ignored, depending on the previous experience with a particular drug. The National Cancer Institute (NCI) Cancer Therapy Evaluation Program has published criteria specifically for reporting adverse effects, including adverse hepatic events, caused by anti-cancer agents. These criteria can be seen in table 1.5 and describe corresponding levels of LFTs with varying grades of toxicity.

Table 1.5: Grades of liver test abnormalities

Common Toxicity Criteria, Version 2.0, from NCI Cancer Therapy Evaluation Program. ULN= upper limit of normal; WNL= within normal limits LLN= lower limit of normal

Liver Tests	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Alkaline phosphatase	WNL	>ULN– 2.5 x ULN	>2.5- 5.0 x ULN	>5.0- 20 x ULN	>20 X ULN
Bilirubin	WNL	>ULN– 1.5 x ULN	>1.5- 3.0 x ULN	>3.0- 10 x ULN	>10 X ULN
GGT	WNL	>ULN– 2.5 x ULN	>2.5- 5.0 x ULN	>5.0- 20 x ULN	>20 X ULN
Hypoalbuminaemia	WNL	<LLN- 3g/dL	≥2- 3 g/dL	<2 g/dL	-
AST	WNL	>ULN– 2.5 x ULN	>2.5- 5.0 x ULN	>5.0- 20 x ULN	>20 X ULN
ALT	WNL	>ULN– 2.5 x ULN	>2.5- 5.0 x ULN	>5.0- 20 x ULN	>20 X ULN

Acute liver injury may be detected by elevation of ALT and AST usually below 10 times the upper reference limit (ULN) with an associated increase of ALP no more than 3 times the ULN (Dufour et al, 2000). The extent of enzymes elevation likely to be predictive of serious hepatotoxicity has been described by Zimmerman and Temple. Hy Zimmerman's rule is based on his observation that drug-induced jaundice caused by severe hepatocellular injury, without a significant obstructive component, has a high rate of bad outcomes, from 10-50% mortality (or transplant) (Zimmerman, 1999). Temple (2006) of the FDA extended this observation from describing jaundice (imprecise) to stating that elevated bilirubin must be to levels of at least 2mg/dl. Therefore the 2 revised requirements for Hy's law are i) evidence that a drug can cause hepatocellular-type injury, generally shown by a higher rate than control of people with 3 x and greater transaminase elevations over the ULN, and ii) cases of increased bilirubin (to at least 2 x ULN) in people with concominant transaminase elevation to at least 3 x ULN and no evidence of intra- or extra-hepatic bilirubin obstruction (Temple, 2006).

Prothrombin time (PT) is the most important predictor of prognosis in comparison to the liver enzyme test; PT cut off times of greater than 4 seconds beyond control or greater than 20 seconds or an international normalised ratio of >6.5 identify patients

at high risk of death. After toxic liver injury, PT values peak by 24-36 hrs after injury and then rapidly return to normal. In paracetamol injury, a persistent increase in PT 4 days after drug ingestions is indicative of liver failure (Dufour, 2000).

In summary, the LFTs as outlined above are routinely used to provide an indication of the condition of the liver in patients with liver disease or suspected hepatotoxicity. Whilst the drawbacks of these tests have been described, LFTs are relied upon in the treatment of patients throughout the world, and can be performed in conjunction with other clinical investigations if further investigation is required. Other investigations include performing other biochemical tests, liver ultrasound scans, computerised tomography scans (CT), magnetic resonance imaging (MRI) scans, or taking a liver biopsy.

1.2 HEPATOTOXICITY

There are several causes of hepatotoxicity which include drugs, environmental chemicals, herbal remedies, and dietary supplements. Drug-induced hepatotoxicity is seen frequently with significant medical, economic, legal, and regulatory implications, and represents a major challenge for clinicians, the pharmaceutical industry and regulatory agencies (Gunawan and Kaplowitz, 2004). Drug-induced hepatotoxicity is the leading cause of failures in drug development (Kaplowitz, 2005) and is the most frequent reason reported for the withdrawal of an approved drug from the market (Lee, 2003). Therefore pre-clinical testing and pre-approval clinical trials do not always detect the hepatotoxic potential of a drug.

The liver is prone to toxicity because it is the first organ perfused by substances absorbed through the gastro-intestinal tract and is actively involved in metabolism of many substances. Several adverse hepatic reactions require metabolism of the compound to reactive metabolites before toxicity occurs (Park et al, 2005). The liver also carries out many detoxifying metabolism reactions and therefore damage to the liver can result in impaired metabolism and accumulation of toxic compounds (Jaeschke et al, 2002). The manifestations of hepatotoxicity, specific mechanisms by which drugs can cause toxicity to liver, factors which affect the susceptibility of an

individual to toxicity, and ways in which hepatotoxicity is screened for during clinical development, will be considered.

1.2.1 Types of Liver Damage Caused by Drugs

Drug-induced liver injury can present as any of the spectrum of acute and chronic liver diseases. A brief outline of the most common forms of liver disease is provided below, and includes hepatitis, steatosis, cirrhosis, cholestasis, and hepatocellular carcinoma (Ishak and Zimmerman, 1995; Kaplowitz, 2005). Hepatitis is defined as inflammation of the liver, and is characterised by jaundice, fever, liver enlargement and abdominal pain (Kaplowitz, 2005). Liver inflammation may result in liver cell damage and destruction, and presence of inflammatory cells in the liver. Acute hepatitis may show up in LFTs as a marked increase in aminotransferases, coinciding with hepatocellular necrosis (Holt and Ju, 2006). Steatosis refers to the accumulation of fat in the liver, and may be accompanied by inflammation and fibrosis. Cirrhosis is a type of chronic, progressive liver disease in which healthy liver tissue is destroyed and results in fibrotic scar tissue. The scar tissue that forms is not able to function and results in non-functioning scar tissue surrounded by functioning liver tissue, therefore the number of functional hepatocytes is reduced, and can obstruct the flow of blood through the portal vein (Gibson and Skett, 2001 pg 140). Cholestasis may be caused by impairment of bile secretion, an obstruction of bile flow, or a combination of the two. As a result of the stoppage of bile flow, bilirubin can enter into the blood and accumulate. Intra-hepatic accumulation of bile acids and excretion products may also occur promoting further hepatic injury (Jaeschke et al, 2002). Drug-induced cholestasis can be subdivided into pure 'bland' cholestasis as described, or 'cholestatic hepatitis' in which liver injury and inflammation cause significant ALT elevations as well as cholestasis and the type of injury that occurs depends on the agent in question. Cholestasis is characterised by jaundice along with an elevation in ALP, conjugated bilirubin, and GGT (Holt and Ju, 2006). Cancer of the liver may be either primary or secondary as metastases from primary cancer. Hepatocellular carcinoma (hepatoma) is the most common form of primary liver cancer, and arises from hepatocytes. Toxicologists have proved extremely efficient in eliminating drug candidates that are carcinogenic from clinical development and

consequently drug-induced hepato-carcinogenesis is not common. It should be noted that these disease states do not always exist in isolation, and the presence of one form of liver disease may lead to another.

Experimental and clinical observations have reached general agreement that there are two main categories of substances that cause hepatic injury: agents that are intrinsically hepatotoxic and cause toxicity to which most exposed individuals are susceptible; and agents that cause hepatotoxicity only in unusually susceptible individuals, which is termed idiosyncratic hepatotoxicity (Zimmerman, 1999). Further consideration will be given to hepatotoxicity caused by pharmaceuticals, subdivided into dose-dependent or idiosyncratic hepatotoxicity.

1.2.1.1 Dose-dependent

Dose-dependent hepatotoxicity, as its name implies, describes an intrinsic property of the drug and as dose increases so too does toxicity in a high incidence of patients (Zimmerman, 1999). Predictable dose-response curves are produced, and there is usually a short interval between drug exposure and observed toxicity. No drug is safe at all doses, a concept first invoked by Paracelsus, stating that 'solely the dose determines that a thing is not a poison' (discussed in Borzelleca, 2000). This concept is illustrated with paracetamol (acetaminophen), which is safely tolerated in the majority of people who use it at recommended doses and is still responsible for half of the cases of liver failure in the US due to overdose (Navarro and Senior, 2006). Metabolism of paracetamol by cytochrome P450s, and extensive depletion of glutathione is required before hepatotoxicity occurs. Thereafter, the reactive metabolite of paracetamol can bind to cellular macromolecules and can eventually result in lethal damage to hepatocytes.

1.2.1.2 Idiosyncratic

By definition, idiosyncratic drug hepatotoxicity occurs in only a small proportion of individuals exposed to the drug, and is therefore harder to predict than dose-dependent (reviewed in Kaplowitz, 2005). Idiosyncratic hepatotoxicity may occur in between 1 in 1000 and 100000 patients exposed to a drug and whilst rare can be

severe and often fatal (Lee, 2003). Adverse reactions that involve the known pharmacological mechanism of the drug (on-target effect) are not considered to be idiosyncratic (Uetrecht, 2007). Idiosyncratic reactions are not dose-related and can be further divided into immune-mediated allergic, or non-allergic reactions.

Non-allergic idiosyncratic reactions are characterised by the absence of the features of hypersensitivity, and there may be a long latency period between drug administration and indication of hepatotoxicity (e.g. six months). Examples of drugs causing non-allergic idiosyncratic hepatotoxicity include troglitazone, and valproic acid.

Characteristics of allergic hepatotoxicity include the presence of rash, fever, eosinophilia, a relatively short latency (one month or less), the presence of autoantibodies, and rapid recurrence of hepatotoxicity on re-exposure to the drug (Gunawan and Kaplowitz 2004). Examples of drugs causing allergic idiosyncratic hepatotoxicity include diclofenac, halothane, and phenytoin resulting in hepatocellular injury, and amoxicillin-clavulanic acid and erythromycin causing cholestatic injury.

There is a deficiency in the availability of approaches to reliably predict idiosyncratic reactions. Whilst several models are being investigated for the study of idiosyncratic hepatotoxicity there is no ideal model (Uetrech, 2007) and it is unlikely that a system will be found that can detect a toxicity occurring at such a low rate in the population. The low incidence of idiosyncratic hepatotoxic reactions explains why these reactions are not usually detected during preclinical studies or clinical trials and are only detected after the drug is approved and hundreds of thousands of patients are being treated.

1.2.2 Mechanisms of Liver Damage Caused by Drugs

Manifestation of drug-induced liver injury can range from an asymptomatic increase in liver enzymes to fulminant hepatic failure. The pathological conditions occur from cellular damage in some way, as a result of drug exposure. Each drug

associated with hepatotoxicity tends to have characteristic latency, and pattern of injury, and the mechanism may either be related to the chemical properties of the parent drug, or be metabolism-dependent (Kaplowitz, 2005). There are several diverse mechanisms resulting in hepatotoxicity of pharmaceuticals, and an overview of these and the cell death that can occur as a result of damage to cells will be given. In general terms, drug reactions can be classified as either hepatocellular, cholestatic, or mixed (Lee, 1995). Further consideration to hepatocyte damage and cholestasis is given below. If sinusoidal, endothelial or fat-storing Ito cells are targeted veno-occlusive disease or fibrosis may occur.

As previously mentioned, the hepatocyte is the most abundant cell type in the liver, and many hepatotoxic drugs first result in damage to hepatocytes (Lee, 1995). Injury to hepatocytes can result either directly from the disruption of intracellular function or membrane integrity, or indirectly, from immune-mediated membrane damage. At least six mechanisms that primarily involve hepatocytes result in liver injury, and the manner in which intracellular organelles are affected defines the pattern of the disease (Lee, 2003).

Drugs may cause hepatotoxicity by disruption of intracellular calcium homeostasis or metabolism of drugs by CYP450s may lead to reactive metabolites capable of covalently binding to intracellular proteins. These insults can lead to intracellular dysfunction, loss of ionic gradients, decline in ATP levels, disassembly of actin fibrils at the hepatocyte surface, blebbing of the cell membrane, cell swelling and cell lysis (figure 1.4 A) (Lee, 2003).

Other drugs may affect the biliary system and can result in jaundice. Bile secretion depends on the function of membrane transporters in hepatocytes and cholangiocytes and on the structural and functional integrity of the bile-secretory systems (Trauner et al, 1998). Disruption of the transport proteins at the hepatocyte canalicular membrane (figure 1.4 B) by binding to and disabling of membrane transporters involved in the export of bile from hepatocytes into bile ductules can therefore interrupt bile flow, resulting in cholestasis (Lee, 2003). Disruption of actin filaments

may occur next to the canaliculus, the specialized portion of the cell responsible for bile excretion (Trauner et al, 1998) and the loss of villous processes combined with the interruption of transport pumps such as multidrug-resistance–associated protein 3 (MRP3) prevent the excretion of bilirubin and other organic compounds (Trauner et al, 1998). Damage to bile ducts or canaliculi can result in cholestasis without marked damage of hepatocytes (Lee, 1995). If cholangiocytes are injured, a likely outcome is prolonged or permanent cholestasis (Lee, 2003). In mixed forms of hepatic injury, combined failure of canalicular pumps and other intracellular processes allow toxic bile acids to accumulate, resulting in secondary injury to hepatocytes (Jaeschke et al, 2002).

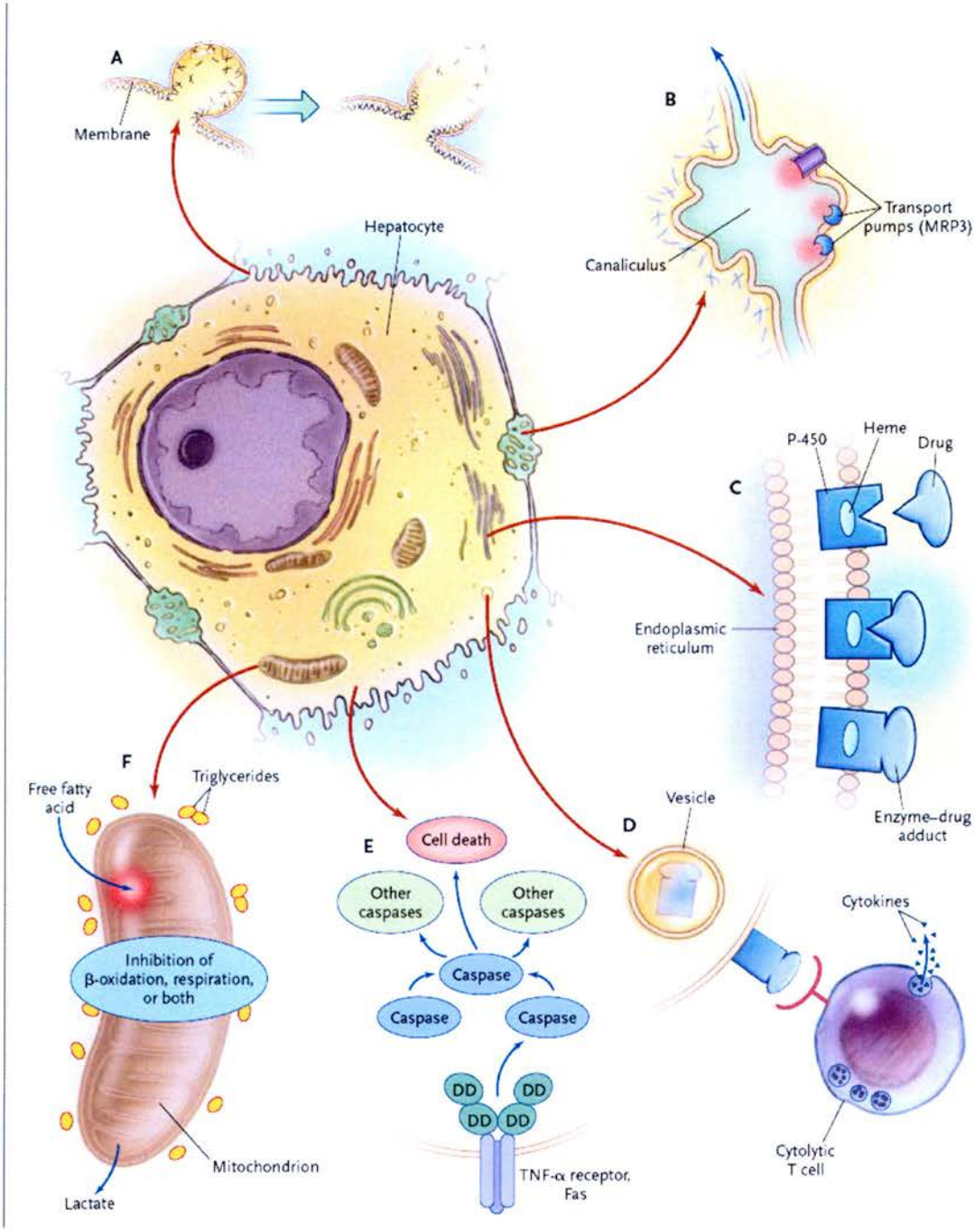


Figure 1.4: Mechanisms of Liver Injury (reproduced from Lee, 2005)
Injury to liver cells occurs in patterns specific to the intracellular organelles affected. The normal hepatocyte shown in the centre of the figure may be affected in at least six ways, labeled A to F. DD denotes death domain.

Drugs are relatively small molecules and therefore are unlikely to evoke an immune response (Lee, 2003). As discussed earlier, the metabolic capability of the liver can result in the production of toxic and non-toxic metabolites, and many hepatocellular

reactions involve the CYP450 system (figure 1.4 C). CYP450-mediated metabolism can lead to the covalent binding of drug to enzyme, thus creating new, nonfunctioning adducts. These enzyme–drug adducts migrate to the cell surface (figure 1.4 D) in vesicles to serve as target immunogens for cytolytic attack by T cells, stimulating an immune response involving both cytolytic T cells and cytokines (Lee, 2003).

In several cases, injury caused by drugs is initiated by bioactivation of drugs in hepatocytes to chemically reactive metabolites. Bioactivation reactions involving CYP450-mediated metabolism leads to reactive metabolites that can deplete reduced glutathione, and become covalently bound to protein and other cellular macromolecules, such as lipids and nucleic acids, with consequent protein dysfunction, lipid peroxidation, DNA damage, or oxidative stress (Kaplowitz, 2001; Holt and Ju, 2006). These chemical consequences can directly affect organelles, including mitochondria, cytoskeleton, endoplasmic reticulum, microtubules, or nucleus, or indirectly influence these organelles through activation or inhibition of signalling kinases, transcription factors, and gene expression profiles (Kaplowitz, 2001). Covalent binding to proteins has been implicated in hepatic toxicity caused with several drugs, such as paracetamol, halothane and diclofenac (Pumford and Halmes, 1997).

Apoptosis can be involved in immune-mediated injury, with the involvement of tumor necrosis factor α (TNFα) and Fas pathways resulting in the cascade of intercellular caspases (figure 1.4 E) and controlled hepatocyte death (Lee, 2003). Further consideration is given to the process of apoptosis below as apoptosis can occur as a result of several cellular insults and is also the mechanism of action by which many anticancer agents are cytotoxic.

A further mechanism of drug-induced liver injury involves damage to mitochondria resulting in disruption of fatty-acid oxidation and/or energy production (Lee, 2003; Holt and Ju, 2006). Certain drugs inhibit mitochondrial functions including β-oxidation (affecting energy production by inhibition of the synthesis of nicotinamide

adenine dinucleotide and flavin adenine dinucleotide, resulting in decreased ATP production) and the respiratory-chain enzymes (figure 1.4 F). Free fatty acids cannot be metabolized, and the lack of aerobic respiration results in the accumulation of lactate and reactive oxygen species. This pattern of injury is characteristic of a variety of agents, including nucleoside reverse-transcriptase inhibitors, which bind directly to mitochondrial DNA, as well as valproic acid, and tetracycline (Lee, 2003).

The outcome of hepatic cell dysfunction may be either triggering of necrotic or apoptotic processes, or initiation of innate or adaptive immunological reactions which may ultimately result in apoptosis (Holt and Ju, 2006). Although the causes of cell death are distinct from one another, apoptosis and oncosis participate in the pathogenesis of the majority of liver diseases, and may be triggered by a number of insults, arising either intra-cellularly or extra-cellularly (Kaplowitz, 2001). The first leads to a pathological pattern of necrosis *in vivo*, with evidence of inflammatory response. Conversely, apoptosis follows a specific pattern of protein activation leading to an orderly cell death and minimising inflammatory response. There is increasing evidence that apoptosis and oncosis are not separate entities but can be initiated by similar initiating signals, the mechanisms of cell death being ultimately related to the level of injury and extent of ATP depletion (Lemasters, 1999). Some drugs may cause cell death by more than one mechanism: for example paracetamol-induced hepatocyte cell death has been linked to both oncosis and apoptosis (Kon et al, 2004).

Despite being morphologically distinct, apoptosis and necrosis can share similar initiating events (death receptor ligation, chemicals, drug-induced hypoxia, oxidative stress), and usually involve the participation of mitochondria (Kaplowitz, 2001). The pattern of cell death depends on the extent of mitochondrial damage, which either leads to rapid ATP depletion, swelling and lysis (oncosis), or a more selective release of cytochrome c in the presence of sufficient ATP to activate executioner caspases (apoptosis) (Jaeschke et al, 2002; Kaplowitz, 2001).

1.2.2.1 Apoptosis

Apoptosis is a cell-suicide mechanism, which eliminates individual cells when they are no longer needed or have become seriously damaged (Ashkenazi and Dixit, 1999). It was first described by Kerr et al in 1972, and is characterised by particular morphological and biochemical changes within the cell. Morphological changes include chromatin aggregation, nuclear and cytoplasmic condensation and partition of cytoplasm and nucleus into membrane bound vesicles called apoptotic bodies. Biochemical changes include phosphatidylserine externalisation, loss of mitochondrial membrane potential, and intranucleosomal cleavage. Cell death by apoptosis does not result in an inflammatory response because the cells do not rupture and are phagocytosed by macrophages.

Apoptosis does not result from a single signal transduction pathway, but from a complex cascade of multi-branching pathways, and can be triggered by both extra-cellular and intracellular signals (see figure 1.5). Two broad apoptosis pathways have been defined: the extrinsic (receptor dependent) and intrinsic (receptor independent) pathways, and both pathways can involve the mitochondria. During the death-receptor dependent pathway, ligand binding to a death receptor (e.g. Fas, TNF-R1, DR3, DR4, DR5 or DR6) recruits downstream effectors such as FADD and TRADD, activating caspase-8 which in turn can activate Bid and the mitochondrial-dependent apoptotic pathway, and caspases 3, 6 and 7 (Petak et al, 2001). Mitochondrial-dependent caspase-9 activation increases caspases 3, 6, 7 activation (Petak et al, 2001). TNF-R1 and FasR are the predominant death receptors expressed in hepatocytes (Faubion and Gores, 1999; Jones and Czaja, 1998; Pinkoski et al, 2000), and Fas is expressed in hepatocytes, cholangiocytes, sinusoidal endothelial cells, stellate cells and Kupffer cells (Pinkoski et al, 2000).

Both the intrinsic and extrinsic pathways of apoptosis result in ATP-dependant activation of cysteine-dependent aspartate specific proteases (caspases). Caspases are constitutively expressed in cells (Weil et al, 1996), and active caspases are generated after proteolytic cleavage of the pro-caspase at specific aspartic acid

residues. Two families of caspases exist that are initiator caspases (-2, -8, -9 and –10) and effector caspases (-3, -6 and –7).

Apoptosis, in contrast to oncosis, is an energy-driven process that requires adenosine triphosphate (ATP) (Tsujimoto, 1997). All cells have an absolute requirement for energy in the form of ATP for many biological reactions to maintain cell viability (Crouch et al, 1993). ATP is generated in cells by glycolysis (in the cytosol) and/or oxidative phosphorylation (in the mitochondria). The balance between ATP production and consumption determines cell fate (Leist et al, 1997; Nicotera et al, 1998). Drugs can interfere with ATP production or cause uncontrolled ATP consumption.

Drug-induced liver injury caused by apoptosis of hepatocytes, seems to be mediated in part via the activation of other cells, activated or recruited to the site of damage via inflammatory signals (Holt and Ju, 2006). The liver contains large numbers of both innate and adaptive immune cells, including the largest populations of tissue macrophages (Kupffer cells), natural killer (NK) cells and NK cells with T cell receptors (Holt and Ju, 2006). Kupffer cells are a major source of TNFα which can trigger apoptosis via TNFR1 signalling (Bradham et al, 1998). Paracetamol- and melphalan-induced liver toxicity can result from production of TNFα, interacting with hepatocytes, either via its soluble form (paracetamol) or membrane-bound (melphalan) (Kresse et al, 2005; Laskin et al, 1995). Binding of TNFα to TNFR1 or TNFR2 recruites FADD via the adapter TRADD. TNF receptors also interact with TRAF1 and TRAF2 activating JNK and NF-κB (Bradham et al, 1998). Caspase activation via FADD induces caspase-dependent apoptosis. NF-κB activation upregulates the transcription of both pro and anti apoptotic proteins which modulate cell death. Cytotoxic T lymphocytes and natural killer cells secrete FasL and induce apoptosis in Fas-expressing hepatocytes (Pinkoski et al, 2000). NK cells also induce cell death via oxidative stress (Jaeschke and Hasegawa, 2006). Activation of Fas recruits the death-inducing signalling complex (DISC), comprised of FADD, FAS and pro-caspase 8. Caspase 8 cleaves Bid, inducing cytochrome c release from the mitochondria. Formation of the apoptosome (cytochrome c, APAF-1 and pro-

caspase 9) activates caspase 9 activating mitochondrial-independent caspases (3,6 and 6). Bile acids can activate Fas-signalling to induce cholestatic-related liver injury.

Anticancer drugs may induce both pathways of apoptosis as their therapeutic mechanism of action, and it is known that the apoptotic pathways of anti cancer drug-induced cell death involve mitochondria-dependent activation through Bax and mitochondria-independent activation through Fas (Kim et al., 2002). Cell-type specific triggering of death receptor and/ or mitochondrial pathways upon drug treatment has been reported (Fulda et al, 2001). The death receptors (e.g. Fas, TNF-R1 DR3, DR4, DR5, and DR6) trigger apoptosis upon engagement by their corresponding death ligands (Petak et al, 2001). Activation of signalling pathways occurs following death receptor ligation.

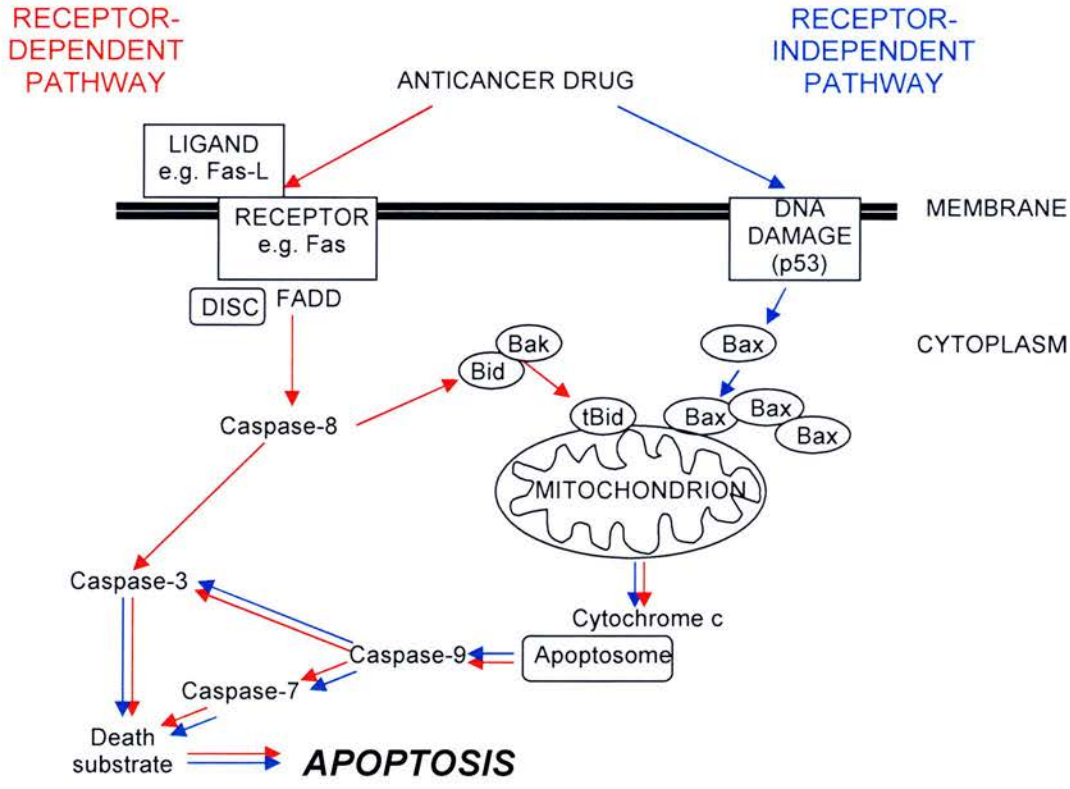


Figure 1.5: Death-receptor-dependent and –independent pathways in anti-cancer drug-induced apoptosis (Adapted from Kim et al., 2002)

1.2.2.2 Oncosis

Oncosis represents a distinctly different pattern of cell death from apoptosis. Necrosis has become an umbrella term for all non-apoptotic accidental cell death (Fink and Cookson, 2005). However the term necrosis is used by pathologists to describe the presence of dead tissue or cells and is actually the sum of changes that have already occurred in cells (Levin, 1998). The term oncosis, however, has been accepted by many investigators as a counterpoint to apoptosis, and is used to describe cells that die by cellular and organelle swelling, blebbing and increased membrane permeability, and is often the consequence of profound ATP depletion (Majno and Joris, 1995; Rosser et al 1995).

1.2.3 Risk Factors for Hepatotoxicity

1.2.3.1 General Risk Factors

A variety of risk factors may impact on the susceptibility of humans to drug-induced hepatotoxicity. These may impact on drug metabolism (see section 1.1.2.3.4) and therefore predispose to toxicity and include age, race, gender, alcohol consumption, pre-existing liver disease, genetic polymorphisms, co-medication, and nutritional status. These factors may be investigated in experiments involving liver tissue from various donor livers. The effect of these factors on host susceptibility to hepatotoxicity appears to depend, at least partly, on the relevance of metabolism of the respective agent to its toxicity (Zimmerman, 1999).

Age can have an important effect on drug metabolism, and therefore alter host susceptibility to hepatotoxicity. Some individuals may show no detectable changes; however most people sustain some decline in liver function with aging. With increasing chronological age liver blood-flow, liver mass and CYP P450-mediated metabolism may decrease. Decreased renal function, increased body fat, polypharmacy, multiple medical problems, and increased likelihood of previous exposure to a particular drug also occur with age and can result in an increased susceptibility to hepatotoxicity (DeLeve, 2003).

Race has an influence on genetic polymorphisms in several drug metabolising enzymes. For example, differences in the phase I drug-metabolising enzyme CYP2D6 between Caucasians and South Pacific Polynesians have been discovered, with poor metabolizer status being higher in Caucasian populations (Wanwimolruk et al, 1998). Another example is the differences in glucuronidation of paracetamol between Caucasians and Chinese that have also been reported (Osborne et al, 1991).

Response of males and females to the same drug can vary. Pharmacokinetics, drug response, and incidence of adverse reactions are not the same between men and women, and drug use is thought to be more frequent in females than males. There are also differences in body composition and size between males and females. One example of this phenomenon is with that anti-cancer agent 5-FU where females are at increased risk of toxicity with 5-FU as drug clearance is slower than in males (Zalcberg et al, 1998).

Genetic polymorphisms of drug metabolising enzymes involved in the metabolism of certain drugs can result in increased hepatotoxicity (Eichelbaum et al, 1992). One example is in the use of irinotecan in patients with Gilbert's syndrome. Irinotecan is excreted in bile as glucuroconjugated SN-38, but in patients with Gilbert's syndrome who have a genetic polymorphism leading to deficiency in UGT1A1 enzyme, irinotecan induces greater toxicity (Floyd et al, 2006). Troglitazone-induced hepatotoxicity has been associated with CYP2C19 but not CYP2D6 polymorphism in the Japanese population, with liver injury occurring more frequently in CYP2C19 mutation carriers (Kumashiro et al, 2003). A further example of genetic polymorphisms that are associated with adverse events is in the use of methotrexate in childhood acute lymphoblastic leukaemia and malignant lymphoma where glutathione-S transferase M1 polymorphism was found to be linked with methotrexate hepatotoxicity (Imanishi et al, 2007).

Any form of pre-existing liver disease can impact on the body's handling of drugs (Andreasen, 1978). For example cirrhosis of the liver, cholestatic jaundice, and liver carcinoma have all been reported to decrease the liver's ability to metabolise drugs,

possibly by decreasing the enzyme activity in the liver, altering hepatic blood flow, or hypoalbuminaemia which leads to lower plasma binding of drugs (Gibson and Skett, 2001 pg 144).

When a patient is treated with more than one drug at the same time, the workload of the liver is increased, and can result in hepatotoxicity. The incidence of adverse drug reactions increases exponentially with the number of drugs prescribed, and drug interactions contribute to this phenomenon. Drug interactions that affect hepatic metabolism may cause toxicity by inhibiting detoxification, inducing activation, or inhibiting biliary excretion (DeLeve, 2003). The use of anticancer drugs in combination regimens and supportive drugs administered during chemotherapy is an example of co-medication and is clinically very relevant.

Excessive alcohol consumption can have a damaging effect on the liver and make it less able to cope with certain drugs. Acute ethanol exposure can decrease drug metabolism (Gibson and Skett, 2001 pg 141). Chronic ethanol exposure can lead to a condition similar to cirrhosis, but firstly can enhance drug metabolism (Gibson and Skett, 2001 pg 142).

Several manipulations of the diet affect the metabolism and toxicity of xenobiotics (Zimmerman, 1999). Intake of protein, dietary fat, and dietary carbohydrate may have an effect. Malnutrition may lead to depletion of tissue-protective molecules such as glutathione. In fasted individuals who have a low hepatic glycogen content, hepatocellular necrosis can result from hypoxia, mitochondrial inhibition and damage to mitochondrial DNA (Jaeschke et al, 2002). Vitamin (retinol [A], riboflavin [B2], thiamine [B1], ascorbic acid [C], and E) and mineral intake may also have an influence. Grapefruit juice is a known CYP3A4 inhibitor, therefore consumption of grapefruit may decrease CYP3A4 metabolism of drugs

1.2.3.2 Risk Factors for Hepatotoxicity in Patients with Cancer

In addition to the risk factors for hepatotoxicity described above, additional factors in some patients with cancer may increase the likelihood of a hepatotoxic reaction, or

make it more difficult to detect. The selection of a chemotherapeutic regimen for treating cancer is based on a thorough assessment of potential hazards regarding the patient's clinical condition and the toxicity of chemotherapy (Sachs et al, 2002).

Several factors can increase the risk of drug-induced liver toxicity in patients with cancer, and examples of these are considered below. They include: the presence of liver metastases; pre-existing liver disease (e.g. viral hepatitis); immunosuppression; and drug combinations. Anticancer drugs are routinely administered in combination regimens, potentially increasing the chance of toxicity caused by drug interactions. Furthermore, anti-cancer chemotherapy is almost always administered in conjunction with other supportive agents. A further complication in attributing hepatotoxicity to a particular anticancer drug is that many of the drugs are given cyclically rather than continuously.

The presence of cancer in the liver can increase the risk of hepatotoxicity with certain drugs. The hepatotoxic potential of platinum derivative oxaliplatin in combination with topoisomerase I inhibitor irinotecan is increased in patients with colorectal cancer treated with neoadjuvant chemotherapy prior to liver surgery. Steatosis is typically reported for both compounds (Zorzi et al, 2007).

Cells of the immune system are rapidly dividing cells, and therefore are targeted by several anti-cancer agents. In patients with underlying hepatitis B and C viruses, chemotherapeutic immunosuppression has resulted in reactivation of hepatitis viruses (Hoofnagle et al, 1982). As a consequence of reactivation of hepatitis B virus infection, fulminant hepatitis has been reported following discontinuation of low-dose methotrexate therapy (Flowers et al, 1990).

There are several treatment-regimes used in the management of cancer, which include a combination of two or more anticancer agents administered concurrently. These regimes are intended to produce a greater cytotoxic effect, but with this comes the increased risk of toxicity and a higher work-load for the liver. Combination regimens can affect toxicity, as each anticancer drug has its own mechanism of

action and toxicity profile, and furthermore these may interact. Examples of combinations that result in increased toxicity compared to administration of the agents in isolation include methotrexate with 6-MP (Floyd et al, 2006), gefitinib with anastrozole (Carlini et al, 2006), and cyclophosphamide with methotrexate and 5-FU (Floyd et al, 2006).

Co-administration of anticancer agents with other therapeutics to treat or prevent the common side effects associated with a particular drug or drug combination is extremely common. Examples include steroids (e.g. dexamethasone), antiemetics (e.g. domperidone, ondansetron, grandisteron), antibiotics (e.g. co-amoxiclav, clarythromycin), anti-fungal agents (e.g. fluconazole, ketoconazole). There have been rare case reports of hepatotoxicity following these drugs, such as jaundice with ondansetron (Vermill and Judson, 1994). If hepatotoxicity is detected following chemotherapy consideration should be given to the co-administered drugs when determining the cause and possible interactions.

1.2.4 Testing for Drug-Induced Hepatotoxicity

Before a drug is approved for use in humans it must go through several years of testing. The Medicines and Healthcare products Regulatory Agency (MHRA) is the government agency in Britain who are responsible for ensuring that drugs meet acceptable standards of safety, quality and efficacy following their development before a licence is granted for use in humans. The Food and Drug Administration (FDA) in America carries out a similar role. There are several factors that may halt the development of a drug at any stage of the drug development process, and prevent the granting of a license for its use in humans. Drug hepatotoxicity is a leading cause of failures in drug development at the clinical phases of investigation (Kaplowitz, 2005). Pre-clinically, there may be indication of hepatotoxic potential of a drug, detected either via *in vitro* tests or in the animal toxicology studies required before a drug is approved for trial in humans. During the various stages of clinical development in patients, liver toxicity may be detected, and can manifest as any of the spectrum of asymptomatic abnormalities in liver enzymes through to life-threatening liver failure. If severe hepatotoxicity occurs in early phase clinical trials, it may stop the clinical development of a drug. Determination of the cause of

toxicity is important, as once the offending article has been identified and withdrawn, liver injury may resolve. An overview of the drug development process and ways in which hepatotoxicity can be detected throughout this process is given below.

Models used to represent the liver in pre-clinical studies include isolated perfused livers, tissue slices, isolated cells or cell organelles, membranes or enzymes, each offering their own advantages and disadvantages (Jaeschke, 2003; Wang et al, 2002; Olinga et al, 1997; Li et al, 1999; Holownia and Brasko, 2004; Li et al, 1999; Stoff-Khalili et al, 2006; Davila et al, 1998, Hewitt et al, 2001; Butterworth et al, 1989; Gomez-Lechon et al, 2003; Ulrich et al, 1995; Anderson et al, 1996; Biagini et al, 2006; Kikkawa et al, 2006; O'Brien et al, 2006; Xu et al, 2004). Whilst these models are often used for studies of drug metabolism, it is not a requirement to perform toxicity testing using these models routinely. An isolated liver preparation for *in vitro* experiments is an extremely useful research tool, and all of these models (with the exception of whole organ) can be obtained from humans and from a variety of animal species. The majority of human liver made available for research is in the form of surgical biopsies, which are usually obtained from patients undergoing partial hepatectomy or from the surgical resection of adult livers prepared for paediatric recipients (Bayliss & Skett, 1996). Models used to predict for toxicity in human liver during the preclinical development of compounds will be given greater consideration below, as they form an important part of the studies presented in this thesis. *In silico* models may be used even before testing in cells or in addition to, to predict hepatotoxic potential of drugs from predefined parameters including structural features. When comparing the models in terms of advantages and disadvantages, factors to be considered include cost, ease of use, ethics, and predictive value of the model. Several of these models have been used previously for assessment of the hepatotoxic potential of many classes of drugs, however a direct comparison of the benefit of these models in predicting anticancer drug induced hepatotoxicity has not previously been made.

1.2.4.1 Isolated enzymes, organelles, and subcellular fractions

It is possible to isolate fractions from the liver for preclinical drug metabolism and hepatotoxicity evaluation, as described below. Liver microsomes may be used to study drug metabolism, and plasma membrane vesicles used to study xenobiotic transport. The metabolic reactions of drugs studied in microsomes are restricted to phase I metabolism and glucuronidation reactions and may provide misleading results with respect to metabolism of a drug.

1.2.4.2 Isolated cells *in vitro*

Cell preparations may be made from a piece of liver tissue isolated from either humans or animals. Isolated human hepatocytes appear to be a suitable experimental model for the study of liver toxicity (Bayliss & Skett, 1996; Gomez-Lechon et al, 2003; Butterworth et al, 1989). Primary cultures of hepatocytes are widely used for studying drug metabolism and toxicity, with a wide variety of *in vitro* assay techniques used to detect the experimental endpoint.

Enzymatic hepatocyte isolation from animal liver was first introduced in 1967 (Bayliss & Skett, 1996). The preparation of isolated hepatocytes from heterogeneous liver tissue involves several steps. Initially the cells must be dissociated from the fibroconnective skeleton. Tissue dispersion requires the disruption of reticulin fibrils along with adhesion proteins, such as fibronectin and laminin, which constitute the framework of the liver lobule. Hepatocyte isolation also requires the disruption of cell-cell adhesion, i.e. the junctional complexes and finally the recovery of the hepatocyte preparation for culture (Bayliss & Skett, 1996).

Human hepatocytes in culture have been shown to maintain plasma protein production, glycolysis, and urea synthesis for periods of several days (Chen et al, 1998). Conversely, drug metabolising enzymes (particularly the cytochrome P450s) decline with time (Bayliss & Skett, 1996). Hepatocyte survival can be extended considerably when cells are maintained in monolayer culture conditions in the presence of an extra-cellular matrix and/or supplemented media. This is necessary as

hepatocytes in suspension, with no attachment to any form of extra-cellular matrix, are viable for only a few hours (Bayliss & Skett, 1996). Therefore, a number of factors, including the extra-cellular matrix, seeding densities, culture media and media supplements need to be considered when culturing human hepatocytes. There are several limitations to the use of fresh hepatocytes, including inability to proliferate, and the eventual loss of some liver functions in culture (Anderson et al, 1996). Many functions such as drug-metabolising enzyme activities decline within the first 24-48hours (Paine et al, 2004). Changes in hepatocyte gene expression are initiated very early into the well-established hepatocyte isolation procedure with collagenase (Paine et al, 2004). Another problem encountered when working with human liver is the limited and irregular availability of material for research purposes. Another major consideration when using human liver to prepare hepatocytes is the variation in the functional activity between cell preparations (Bayliss & Skett, 1996). This variability may be the result of the length of time taken in obtaining the sample, sample storage conditions, or the sample history, as well as the genetic and environmental factors affecting the donor (Olinga et al, 1998). Information regarding the patient history, dietary habits, and genetic polymorphism is valuable when interpreting results (Bayliss & Skett, 1996).

Cryopreservation of hepatocytes may help to overcome some of the issues relating to supply of hepatocytes for research. It allows cells from one donor to be used over a period of time and to be used for testing more than one compound. It also ensures that this valuable and finite resource is not wasted if all of the cells isolated are not needed immediately. The conditions necessary for successful cryopreservation continue to be improved on (Ilan, 2002) helping to ensure viable and plateable cells after thawing. They therefore offer practical advantages, however their limitations should not be forgotten. Reports mainly focus on drug metabolism studies as opposed to hepatotoxicity studies following freezing.

In addition to the use of isolated hepatocytes on their own, these cells may be co-cultured with other different cell types e.g. Kupffer cells.

As well as isolated hepatocytes, which are a limited resource, there are several cell lines that are immortalised and therefore able to replicate *in vitro* that are used to study the liver. Immortalised, stable, differentiated liver cell lines, capable of proliferation, and possessing liver-specific function are of great value for the *in vitro* study of the human liver. These cell lines have been developed to overcome some of the problems encountered with normal tissues *in vitro*, and offer the advantages of replication *in vitro*, ready availability, and no concerns about inter-individual variability that are observed in patient samples (Stacey et al, 2001). These fall into two categories: those that have specifically been immortalised by addition of a viral vector, and those that are derived from liver cancer and naturally proliferate.

A replicative culture of engineered immortalised adult human liver epithelial cells (hepatocytes) have been produced by Pfeifer et al (1993) by transfection of hepatocytes with recombinant simian large T antigen virus 40 (SV40) DNA, and are called THLE-2 and THLE-3. These cells have been shown to undergo over 100 population doublings, express several hepatocyte markers including cytokeratin 18 and albumin, and have functional phase I and II drug metabolising enzymes, such as cytochrome P450s and glutathine-S-transferase (Pfeifer et al, 1993). Screening for hepatotoxicity by Bristol-Myers Squibb Pharmaceutical Research Institute (Dambach et al, 2005) incorporated studies of immortalised hepatocytes established by Pfeifer et al.

Hepatocellular carcinoma (hepatoma) cell lines are established from cancers that develop from hepatocytes. Hepatocellular carcinoma is the most common type of primary liver cancer, accounting for over 85% of all liver cancers (CancerHelp UK). Examples of human hepatoma cell lines, which were established from primary hepatomas, include HepG2, Huh-7 D12, Hep3B, PLC/PRF/5, and SK-HEP-1 and are available from the American Type Culture Collection (ATCC). These cell lines were shown to retain some of the specialised functions of the liver that are normally lost by primary hepatocytes in culture, such as secretion of some major plasma proteins, and expression of a wide range of phase I and II drug metabolising enzymes (Mersch-Sundermann et al, 2004; Wilkening et al, 2003). However, these cells are tumorigenic, have lost their contact inhibition, and therefore differ from fresh

hepatocytes and immortalised hepatocytes. Many studies of the hepatotoxic potential of drugs have included Hep G2 cells (Brandon et al, 2005; Burczynski et al, 2000; Harris et al, 2004; Miret et al, 2006; O'Brien et al, 2000; Samali et al, 1999; Tirmentstein et al, 2000; Yang et al, 2004), and there are also studies including Huh-7, Hep3B and PLC/PRF/5 cell lines.

As mentioned, several *in vitro* assays are available to measure different aspects of cell death and are compatible with the cell types described above. Miret et al (2006) compared different methods to assess cytotoxicity in HepG2 cells and concluded that a selection of assays (ATP, necrosis, and caspase-3/7 activation) used to detect different aspects of cells death is needed to evaluate drug-induced liver injury. Measurement of cellular ATP content was used by Lloyd et al (2002) to detect troglitazone cytotoxicity in human hepatocytes. The measurement of ADP and ATP in cells and calculation of ADP:ATP ratio has been used to distinguish oncosis from apoptosis (Bradbury et al, 2000).

1.2.4.3 Liver slices

Human liver slices are the ex-vivo model with the highest structural integrity in which to study the effects of drugs pre-clinically. Liver slices can also be made from the liver of several species of animal. Liver slices retain the intact structure of the organ, maintaining cellular heterogeneity, and can be as thin as 100μm, however the optimal thickness for slices found to retain their viability during culture is 200-250μm. Therefore, when used as a model, it must be remembered that the drug must penetrate through layers of cells. Endpoint toxicity measurements such as potassium retention, ATP content, enzyme leakage, protein synthesis and MTT reduction have all been used in this system. Cultured, precision-cut rat liver slices were used by Moronvalle-Halley et al 2005) to evaluate drug-induced apoptosis. Liver slices from rat and guinea pig have been used to study the hepatotoxicity of drugs including hepatotoxic compounds such as carbon tetrachloride and halothane respectively, and were found to be predictive of toxicity (Olinga et al, 1997). It is also of interest that liver slices can be cryopreserved, however these are not readily used due to similar problems as with the isolated cells. It is not apparent whether the extra technical, and

therefore cost considerations are justified when compared to testing with fresh hepatocytes.

1.2.4.4 Isolated perfused liver

The liver may be removed following sacrifice of an animal, and perfused so that a compound can be administered to the isolated organ. Isolated perfused liver has been used to study rat liver, but has not been employed for this purpose in humans due to the large size of the organ, limited availability, and ethical considerations (Olinga et al, 1997).

1.2.4.5 Liver *in vivo*

Whole animals are frequently used to study the liver *in vivo*, and may include rodent and non-rodent species. Signs of liver toxicity in the animals should be documented following the administration of the test compound, and if the animals are sacrificed at the end of the experiment, the liver may be removed and examined for signs of liver damage. Advantages of using the whole animals include maintenance of liver structure and drug metabolising capabilities, and the possibility for pharmacokinetic analysis. Disadvantages include the cost, influence from other organs, and endpoint analysis is more complicated. There is also the potential for interspecies differences in drug metabolism to confound the results, although transgenic mice expressing human CYP450 have been developed to try to overcome this (Gonzalez and Yu, 2006).

1.2.4.6 Clinical Trials

Before a drug is granted approval for clinical trials in humans, pre-clinical drug development to examine efficacy and toxicity must take place. This involves testing efficacy and toxicity both *in vitro* and *in vivo*. Investigations into a drug's absorption and metabolism, toxicity of drug metabolites, and excretion as well as genotoxicity studies are carried out. Acute and long-term toxicology studies are usually required in one rodent and one non-rodent animal species. This stage helps plan clinical development strategies and ensures adequate knowledge and safety considerations before trials in humans.

After a drug has met all the necessary requirements to gain approval for use in humans, clinical trials may begin. Phase I clinical trials involve administering the drug to a small number of healthy male volunteers and assessing safety, determining a safe dosage range and determining side effects. Following successful phase I development the drug will enter phase II trials during which it will be administered to patients suffering from the condition the drug is intended to treat, and will examine efficacy and safety. Phase III trials follow on from successful phase II trials and extend into a much larger patient population looking to confirm efficacy, monitor side effects, and compare with other treatments. At all stages adverse drug reactions, including hepatotoxicity will be closely monitored for, and LFTs may be performed during any phase of clinical trials to assess if the new compound is having adverse effects on the liver. A drug will be reported as hepatotoxic if it causes significant changes in LFTs.

Following successful clinical trials, in terms of efficacy and lack of toxicity, a drug will be granted approval for use in patients. Phase IV trials, also known as post-marketing surveillance, describe the process of monitoring a drug once it has been licensed. The occurrence of hepatotoxicity of drugs post-marketing can result in drug withdrawals, modifications of use, and warnings (Kaplowitz, 2005; Ballet, 1997). Several drugs do make it through the full development process, which lasts on average 12 years, and are then withdrawn or their use restricted following marketing. This is not only extremely costly for the pharmaceutical company, but is as a result of serious or fatal adverse events. Any steps towards reducing this are therefore worthwhile.

1.2.4.7 Testing for Drug-Induced Hepatotoxicity in Oncology

An overview of the drug development process for other classes of drugs is given above. There are several key differences in the way the pre-clinical and clinical development process is carried out when the drug indication is oncology, and these are outlined below.

Preclinical testing strategies may involve any of the *in vitro* and *in vivo* models described above, and in addition testing will be performed in cancer cell lines and implanted tumours in animals. A summary of the regulatory considerations for pre-clinical development of anticancer drugs in the US is provided by DeGeorge et al (1997). In the UK, preclinical *in vivo* safety testing of anticancer agents need only be carried out in rodents. Following an evaluation of rodent-only toxicology studies by Newell et al (1999), Cancer Research UK in conjunction with the European Organisation for the Research and Treatment of Cancer (EORTC) promote minimum preclinical toxicology assessment, using no non-rodent species (Cancer Research UK website).

For ethical reasons, phase I first-in-human trials of novel anti-cancer agents are undertaken in patients with cancer who are generally highly pre-treated and unresponsive to all other treatments for their type of cancer. Therefore they are assumingly resistant to other anti-cancer therapies. Phase I trials can be conducted in patients with any kind of cancer. Following successful phase I trials, the compound will progress into phase II trials in a particular cancer type, and phase III studies follow successful phase II studies in the same way as for other classes of agents.

1.3 HEPATOTOXICITY INDUCED BY ANTICANCER AGENTS

As mentioned previously, the exposure of individuals to many different classes of drugs, including several anti-cancer agents, can result in liver toxicity. Traditionally anti-cancer drugs are administered at the maximum tolerated dose and have a narrow therapeutic index (Colombo et al, 2001), increasing the potential for toxicity. In oncology, the aim of treatment with cytotoxic drugs is to selectively target and kill all cancerous cells, whilst leaving the healthy tissue unharmed. In practice, anti-cancer drugs induce cytotoxic effects in all proliferating cells and, to a certain extent, non-dividing cells. For some anti-cancer drugs this manifests as liver-related dose-limiting toxicity. Serious hepatotoxic reactions following chemotherapy are fortunately infrequent; however some compounds do make it through preclinical safety testing and cause toxicity during clinical trials. Drug-induced liver injury is a

major reason for failure of novel anticancer agents in clinical development (Spicker et al, 2007). Therefore, further research into the detection and understanding of which anti-cancer drugs are likely to cause hepatotoxicity is required.

1.3.1 Chemotherapy-induced hepatotoxicity

There are numerous case reports, papers, and review articles that have been published documenting clinical signs of liver toxicity following administration of anti-cancer agents to humans. The chemical properties and mechanism of action of these agents may aid understanding of the hepatotoxicity caused; however route of administration and risk factors (discussed in section 1.2.3) may also alter risk of hepatotoxicity.

1.3.1.1 Type of chemotherapy (single-agents)

Drugs from most of the classes of anticancer agents have been reported to cause hepatotoxicity and include DNA damaging agents, antimetabolites, topoisomerase inhibitors, microtubule-targeting drugs, and antitumour antibiotics. Table 1.6 reports hepatotoxicity resulting from drugs administered as single agents. Dose-dependent toxicity and off-target effects play a significant role in liver injury. Hepatotoxicity is reported when there are significant alterations in LFTs. However, cytotoxic agents commonly cause transient LFT abnormalities, without clinical evidence of liver impairment. Mild or moderate transient elevations of LFTs without other signs of liver toxicity could often be ignored. However, when irreversible hepatotoxicity occurs, early detection of this and withdrawal of the offending drug is imperative (DeLeve, 2003).

Anticancer agents are reported to cause cell death in cancer cells by induction of apoptosis. It is known that the apoptotic pathways of anti cancer drug-induced cell death involve mitochondria-dependent activation through Bax and mitochondria-independent activation through Fas (Kim et al, 2002). A cell-type specific triggering of death receptor and/or mitochondrial pathways upon drug treatment has been

reported (Fulda et al., 1998, 2001). This may be of interest when examining the way in which an anticancer agent causes hepatotoxicity or induces liver cell death.

Table 1.6: Effect of chemotherapy on LFTs and liver injury, when used as single agents

Drug reported to cause hepatotoxicity	Liver injury	Liver test abnormalities	References
6-mercaptopurine	Hepatocellular/ cholestatic liver disease, jaundice	Elevated bilirubin, aminotransferase and alkaline phosphatase	Adamson et al., 1990, 1992; Berkovitch et al., 1996; Laidlaw et al., 1995
6-thioguanine	Case reports of VOD, may be reversible upon discontinuation of drug	-	Satti et al., 1982
α-interferon	-	Dose-related mild transaminitis	Quesada et al, 1986
Azathioprine	Cholestasis, VOD	ALP, bilirubin and GGT elevations	DeLeve et al, 1996; Evans et al., 2001; Floyd et al, 2006.
Busulfan	Case reports of cholestatic liver injury, jaunidice, VOD	-	Underwood et al., 1970, 1971; Morris and Guthrie, 1988.
Carboplatin	-	Reversible enzyme and bilirubin elevations	Hruban et al., 1991
Carmustine (BCNU)	Case reports of liver injury, some fatalities	Up to 25% of patients	De Vita et al., 1965;
Chlorambucil	Reports of jaundice		Koler and Forsgren, 1958
Chlorozotocin	Case reports of severe cholestatic liver injury	Up to 25% elevations of aminotransferases	Belt et al., 1980; Hoth et al., 1978
Cyclophosphamide	Rare case reports, not routinely observed, high dose VOD	Uncommon	Aubrey, 1970; Goldberg and Lidsky 1985.
Cyproterone acetate	8 case reports of hepatocellular injury with 4 fatalities	10% with alkaline phosphatase and 3% with aminotransferase elevations	Hinkel et al., 1996
Cytarabine (Cytosine arabinoside, ara-C)	Case reports of cholestatic jaundice	Transient enzyme abnormalities at high dose	Donehower et al, 1986; George et al, 1984, Pizzuto et al, 1983
Dacarbazine (DTIC)	VOD	Mild transient elevations in aminotransferases in up to 50%	Asbury et al, 1980; Ceci et al, 1988
Dactinomycin	Slight steatosis, VOD	Increased aminotransferases	Pritchard et al., 1989

Drug reported to cause hepatotoxicity	Liver injury	Liver test abnormalities	References
Doxorubicin	Rare reports of steatosis	Increased aminotransferases and bilirubin	Aviles et al., 1984
Etoposide	Case reports of hepatocellular injury	Low incidence of moderate, transient LFT abnormalities	Johnson et al, 1983; Tran et al., 1991
Fluorodeoxyuridine	Hepatitis	Transaminases, ALP and bilirubin elevations	Doria et al, 1986
Flutamide	Reported cases of severe cholestatic hepatitis, fatalities	Aminotransferase elevations, hyperbilirubinemia	Wysowkski et al, 1993
Gemcitabine	Case reports of fatal fulminant liver failure, VOD	aminotransferase elevations in 60% of patients	Coeman et al., 2000; Yang at al., 2000; Robinson et al, 2003
Hydroxyurea	Hepatitis	-	Heddle and Calvert, 1980
Interleukin-2 (aldesleukin)	Reversible cholestasis	Hyperbilirubinemia, AST ALT ALP elevations, hypoalbuminemia, prolonged prothrombin times	Fisher et al, 1989; Huang et al, 1990; Nakagawa et al, 1996
L-asparginase	Steatosis (42-87% incidence at autopsy), occasional hepatocellular necrosis.	Abnormalities in >50% patients	Haskell et al, 1969
Lomustine (CCNU)	Rare case reports of liver injury, some fatalities	Uncommon	Floyd et al., 2006
Megestrol acetate	Cholestatis, jaundice	-	Foitl et al., 1989
Melphalan	Not routinely observed	High dose can result in transient enzyme elevations	Lazarus et al, 1983; Pingpank et al., 2005
Mercaptopurine			Evans et al., 2001
Methotrexate	Fibrosis and cirrhosis	Transaminase elevation common at high doses	Mcintosh et al., 1977
Mithramycin	Hepatocellular damage	AST and LDH elevations	Green and Donehower, 1984
Mitoxantrone	-	Transient abnormalities in bilirubin or aminotransferases	Paciucci and Sklarin, 1986.
Oxaliplatin	Case reports of sinusoidal lesions, steatohepatitis		Tisman et al., 2004; Rubbia-Brandt et al., 2004; Fernandez et al, 2005; Arotcarena et al., 2006

Drug reported to cause hepatotoxicity	Liver injury	Liver test abnormalities	References
Paclitaxel	1 case report of fatal hepatic coma in patient with multiple liver metastases	Elevated bilirubin, ALP, AST, ALT	Oettle et al., 2000; Feenstra et al., 1997
Streptozotocin	-	Up to 67% but most patients had liver metastases	King & Perry, 2001
Tamoxifen	Cholestasis, Hepatitis, Steatosis, case report or non-alcoholic steatohepatitis, some with cirrhosis	Elevated enzymes, hyperbilirubinemia	Pinto et al., 1995; Elefsiniotis et al., 2004

1.3.1.2 Route of administration

The hepatotoxic potential of a drug can be modified depending on the route of drug administration. A change in the route of administration, by changing the pharmacokinetic profile of the drug, can increase the incidence of liver toxicity. An example of this phenomenon is in the hepatic intra-arterial administration of a drug. The primary aim of hepatic intra-arterial infusion is to ensure that a higher drug concentration reaches the liver than would occur than if the drug were administered systemically. When 5-FU is administered for the treatment of non-resectable liver metastases from colorectal cancer by delivery into the hepatic artery, a significant advantage in terms of tumour response and median survival time was observed, when compared with systemic administration (intra-venous). The downside of administration of intra-arterial 5-FU is the increased observation of hepatotoxicity (Link et al, 1999). Similarly, intra-arterial floxuridine (FudR) following complete resection of liver metastases caused biliary toxicity (persistently elevated serum bilirubin and alkaline phosphatase) in approximately 35% of cases and one patient developed cirrhosis and died of progressive liver failure associated with this toxicity (Onaitis et al, 2003). One further example is that melphalan infusion has been associated with grade 3–4 hepatotoxicity in 19% of treatments in humans in one phase I trial (Pingpank et al, 2005). As previously discussed, melphalan has been shown to induce liver injury via the expression of membrane-bound TNF in Kupffer cells (Kresse et al, 2005). All of these examples are likely to be caused by a dose effect, whereby toxicity is observed in the liver because a high dose of drug reaches the liver.

When a patient is treated with more than one drug at the same time the workload of the liver is increased, and can result in hepatotoxicity. The incidence of adverse drug reactions increases exponentially with the number of drugs prescribed, and drug interaction contribute to this phenomenon (Blower et al 2005). Drug interactions that affect hepatic metabolism may cause toxicity by inhibiting detoxification, including activation, or inhibiting biliary excretion (DeLeve, 2003). The use of anticancer drugs in combination regimens and supportive drugs administered during chemotherapy is an example of co-medication. Co-administration of anti-cancer agents with other therapeutics to treat or prevent the common side effects associated with a particular drug or drug combination is extremely common. Examples include steroids (e.g. dexamethasone), antiemetics (e.g. domperidone, ondansetron, grandisteron), antibiotics (e.g. co-amoxiclav, clarythromycin), anti-fungal agents (e.g. fluconazole, ketoconazole). There have been case reports of hepatotoxicity following some of these drugs, such as jaundice with ondansetron (Vermill and Judson, 1994). If hepatotoxicity is detected following chemotherapy, consideration should be given to the co-administered drugs when determining the cause and possible interactions.

1.3.2 Hepatotoxicity during clinical development of novel anti cancer agents

The drugs listed in table 1.6 have all been licensed for the treatment of various types of cancer, and much of the evidence of their hepatotoxic potential has arisen during late-phase clinical trials or after licensing. There are also reports of newer anti-cancer agents that have passed pre-clinical testing and demonstrated hepatotoxicity, which may be dose-limiting, during phase one clinical trials. Examples of these can be seen in table 1.7.

Table 1.7: New anti-cancer agents that have had effects on LFTs and/or caused liver injury during clinical trials

Drug reported to cause hepatotoxicity	Hepatotoxicity detectable in animal studies?	Transient liver test abnormalities in humans	References
4-ipomeanol	Detected in birds, but not in hamsters. Mild and subclinical elevation in hepatocellular enzymes in dogs and rodents.	Transaminase elevations, particularly ALT, at doses above 826mg/m^2 (maximal after 4 days, resolved by at least day 22 after treatment).	Rowinsky et al., 1993
8-chloro-cAMP	Not in beagle dogs	¾ patients at top dose (0.15mg/kg/h) developed hyperbilirubinemia	Propper at el., 1999
9-cis-retinoic acid (ALRT1057)	-	Grade 2 transient transaminase elevations.	Adamson et al., 2001
17-allylamino, 17-demethoxygeldanamycin	-	Dose-limiting grade 3-4 elevated AST and/or ALT at 450mg/m^2/week	Banerji at al., 2005
cis-malonato[(4R,5R)-4,5-bis(aminomethyl)-1,3-dioxolane] platinum (II) (SKI 2053R)	-	At 480mg/m^2 2/3 patients developed grade 4 hepatotoxicity, and 1/3 developed grade 2 hepatotoxicity (ALT/AST)	Kim et al., 2001
Clofarabine	(Safe starting dose for humans overestimated from animal toxicology studies)	Transient abnormalities in bilirubin or transaminases. DLT at 55mg/m^2 dose for treatment of acute leukaemia.	Jeha et al., 2004; Kantarjian et al., 2003
Ecteinascidin 743	Detected in rats, particularly female. Suggested bile duct toxicity. Hepatobiliary toxicity in monkeys.	Grade 3 elevated transaminases at doses >216μg/m^2/day in patients. AP elevation in some patients. Resolved within 28 days. Bile duct	Ryan et al, 2001. Donald et al, 2002.
Elsamitrucin	-	Dose-limiting hepatotoxicity. Transient ALT and AST elevations.	Raber et al., 1992
Estramustine phosphate	-	Mild, transient bilirubin and transaminase elevations	Hudes et al., 2002

Drug reported to cause hepatotoxicity	Hepatotoxicity detectable in animal studies?	Transient liver test abnormalities in humans	References
Gemtuzumab	Marked changes in histopathology of the liver of rats and monkeys.	Transient reversible grade 3 or 4 hyperbilirubinaemia, transaminase elevations (AST or ALT). Reports of fatal VOD.	Cohen et al, 2002; Bross et al, 2001.
Spicamycin Analog KRN5500	-	Abnormalities in bilirubin or transaminases	Supko et al., 2003
Squalamine	Detected.	Dose-limiting hepatotoxicity. Reversible hyperbilirubinemia, elevated transaminases, alkaline phosphatase and lactate dehydrogenase.	Bhargava et al., 2001; Hao et al., 2003
Swainsonine	AST elevations in rhesus monkeys, but at doses approximately 16 times higher than dose used in phase I studies.	Dose-limiting hepatotoxicity. Hyperbilirubinemia and elevated transaminases, especially AST.	Goss et al., 1994; Goss et al, 1997.

The purpose of the studies presented in this thesis is to detect hepatotoxicity at an earlier stage of drug development.

Key issues in predictive toxicology are (i) whether *in vivo* responses can be predicted from *in vitro* systems and (ii) whether long-term effects an be predicted from short term ones.

1.4 AIMS AND OBJECTIVES OF THIS STUDY

There are several examples of anti-cancer agents that were not anticipated to cause hepatotoxicity following pre-clinical testing, but have caused hepatotoxicity in humans during clinical trials. More toxicity is accepted with anticancer agents than with other classes of drugs. However, as the number of drugs available to treat the wide range of cancers is increasing, tolerance of severe toxicities should not be necessary. Early detection of hepatotoxicity caused by chemotherapy would be beneficial, and potentially allow unsuitable drugs to be eliminated at an earlier stage of drug development. This would not only benefit patients, but also help to reduce the cost of the expensive drug development process.

The primary aim of this project is therefore to improve early detection of drug-induced hepatocyte toxicity caused by anti-cancer agents, using *in vitro* models including human hepatocytes, immortalised hepatocytes and hepatoma cell lines. The main objectives are:

i) to determine the most appropriate *in vitro* model by considering cell phenotype (morphology, proliferation) and genotype (mRNA expression);

ii) to optimise the most promising model;

iii) to determine the most appropriate *in vitro* model by comparing the response of each cell line to selected anti-cancer agents;

This thesis will examine several *in vitro* models and will first address the intrinsic parameters of the selected models. Hepatocytes have been used for investigation of the hepatotoxic potential of other classes of drugs; however their usefulness in oncology has not yet been fully evaluated. Anti-cancer agents with a known hepatotoxic or non-hepatotoxic profile have been selected for drug treatment of the cells and determination of cytotoxicity. There are numerous assays available to detect cell death *in vitro*, and it is likely that liver cell death induced by anticancer agents will be detected with some of them. DNA damaging agents induce cytotoxic effects; therefore cell death will be considered as the endpoint for evaluating drug-induced hepatotoxicity. Instead of aiming to detect an absence of toxicity, it is more

interesting to determine the difference between an active concentration killing tumour cells and achievable in the clinic, and a toxic concentration, killing hepatocytes. A key strategy is therefore to set up assays that will assess drug-induced liver injury based on the differential between normal and cancer cells. Therefore the cancer type in which the anticancer drugs are intended to treat will also be investigated. The "*in vitro* therapeutic index" may be able to discriminate which drug is likely to induce significant liver toxicity, at predicted therapeutic doses. The contribution of oncosis and/or apoptosis to cytotoxicity, and early detection of cell damage during drug exposure will also be considered using assays to measure caspase activation and ATP levels within drug-treated cells.

This approach is intended to allow the selection of the most appropriate *in vitro* cell model to detect anti-cancer drug toxicity to the liver. Features to be considered include ease of use, cost, reproducibility, and the predictive value of the models. Ultimately, an *in vitro* system that bears the greatest resemblance to the response of human liver to cancer chemotherapy is desired.

CHAPTER 2: Results
Characterisation and Optimisation of Cell Models

2.1 INTRODUCTION

2.2 CELL MORPHOLOGY
- 2.2.1 Hepatocytes
- 2.2.2 Immortalised Hepatocyte Cell Line
- 2.2.3 Hepatoma cell lines

2.3 VIABILITY OF HEPATOCYTES
- 2.3.1 Fresh Human Hepatocytes
- 2.3.2 Cryopreserved Human Hepatocytes

2.4 CELL GROWTH CHARACTERISTICS
- 2.4.1 Hepatoma Cell Lines and Immortalised Hepatocytes

2.5 GENE EXPRESSION OF *IN VITRO* MODELS
- 2.5.1 Quantitative RT-PCR Set-up
- 2.5.2 Reference Gene Selection
- 2.5.3 Expression of Liver-Specific Genes in Fresh Hepatocytes
- 2.5.4 Expression of Liver-Specific Genes in Cryopreserved Hepatocytes
- 2.5.5 Expression of Liver-Specific Genes in Immortalised and Hepatoma Cell Lines
- 2.5.6 Comparison of Gene Expression Between *in vitro* Models
- 2.5.7 Expression of Stress-Induced Genes in Hepatocytes

2.6 OPTIMISATION OF FRESH HEPATOCYTE TRANSPORT
- 2.6.1 Impact of Transport Media on Hepatocyte Viability
- 2.6.2 Impact of Transport Time on Hepatocyte Viability
- 2.6.3 Impact of Transport Time on Hepatocyte Gene Expression
- 2.6.4 Impact of Transport Media on Hepatocyte Gene Expression

2.7 DISCUSSION

2.1 INTRODUCTION

There are several cell types and systems that have previously been assessed for studying liver toxicity, including isolated perfused livers, tissue slices, isolated cells and cell organelles, membranes or enzymes, as described in section 1.2.4. Interspecies differences have been reported for a number of liver enzymes involved in drug metabolism and are of particular importance and relevance when considering the models of choice to represent the liver in these studies. Therefore in this study, solely cells of human origin that may represent hepatocytes were selected. Several *in vitro* models were studied: hepatoma cell lines, an immortalised hepatocyte cell line, fresh hepatocytes, and cryopreserved hepatocytes. The different *in vitro* models were assessed for expression of liver-specific genes required for essential liver-specific functions. Cell morphology, cell growth, and viability were also evaluated to compare the characteristics of the different models. Fresh hepatocytes have been described as the 'gold standard' for *in vitro* testing of drug candidates (Roymans et al, 2004). Therefore, because the hepatocytes used in these studies are sourced externally and transport is a requirement, variables including time and media for their transport were evaluated.

2.2 CELL MORPHOLOGY

Initially cell morphology was examined using light microscopy. Cell morphology and function are closely related characteristics (Wilkening et al, 2003). All of the cell types considered adhere to tissue culture plastic, and form a monolayer culture although some are more adherent than others. Cells were plated in the appropriate media and allowed to attach to tissue culture plastic before photographing. The hepatocytes and immortalised hepatocytes require the presence of an extra-cellular matrix to maintain their morphology in culture (Kudryavtseva and Engelhardt, 2003), and this was incorporated in the study design. The time between plating and photographing varied for the different cell types.

2.2.1 Hepatocytes

The morphology of hepatocytes from all donors was examined under the microscope after plating and at a maximum of 24-hour intervals throughout the course of the

experiments (see figure 2.1 for an example from donor 12). They were strongly attached to the collagen-I pre-coated plastic and formed cell-cell contact. As expected, they were polygonal and had single or double nuclei.

After recovery from liquid nitrogen, the cryopreserved hepatocytes that attach to the plate look similar to the fresh hepatocytes, also being polygonal and having single or double nuclei (see figure 2.2). However, in 1 of the 4 donors studied, following resuspension the cells failed to attach to the tissue culture plastic. Furthermore, when 20000 viable cells that have been cryopreserved are plated per well it appears that fewer cells attach than when 20000 viable fresh cells are plated per well (observations suggested ≤50% cells attached). This suggests that the cryopreservation and thawing process alters the cell membrane and affects cell attachment, and has been reported previously (Garcia et al, 2003; Li et al, 1999).

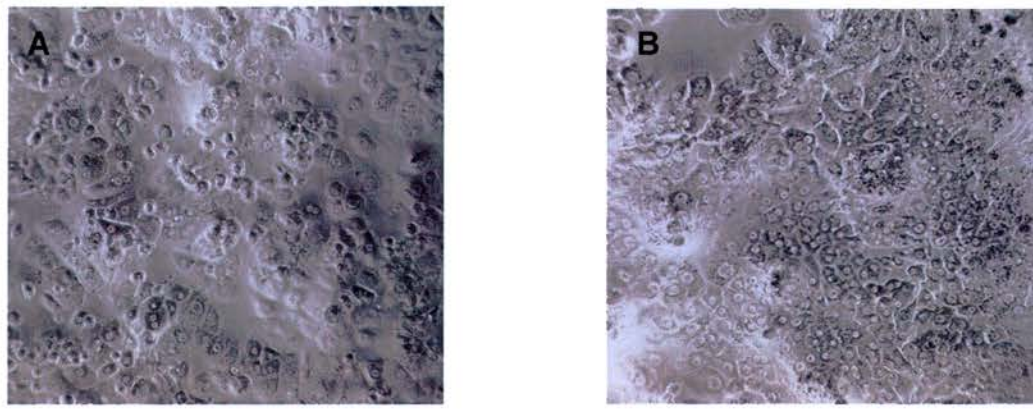


Figure 2.1: Morphological comparison of fresh hepatocytes in living monolayers, 12- and 108- hours after plating

20000 viable fresh hepatocytes (from donor number 12) were plated per well in supplemented WEM and photographed after attachment and before cytotoxicity assays begun (12 hours after plating; A), and at the end of the 96-hour cytotoxicity time-course experiments (108 hours after plating; B). To photograph the cells, media was removed and attached cells photographed using a Diavert microscope at x200 magnification.

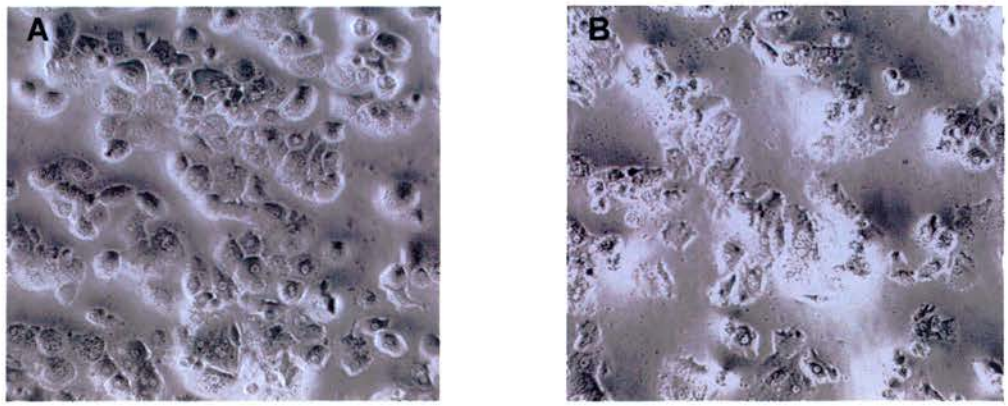


Figure 2.2: Morphological comparison of attached cryopreserved hepatocytes in living monolayers

20000 viable cryopreserved hepatocytes (from donor C3 (A) and donor C4 (B)) were plated per well in supplemented WEM and left to attach (for approximately 6 hours). Media was then removed and attached cells were photographed using a Diavert microscope at x200 magnification.

2.2.2 Immortalised hepatocyte cell line

The immortalised hepatocyte cell line, THLE-2, was grown in a coated tissue culture flask for 2 days before photographing. THLE-2 cells were dissimilar to polygonal fresh hepatocytes, and were more epithelial-like in nature (figure 2.3).

Figure 2.3: Immortalised hepatocyte cell line, THLE-2
One vial of THLE-2 cells (approximately $2x10^6$ cells) was recovered from liquid nitrogen into a T25 tissue culture flask. After 2 days the flask was confluent, and the cells were photographed using a Diavert microscope at x200 magnification.

2.2.3 Hepatoma cell lines

When considering the hepatoma cell lines, there were marked morphological differences not only between the cell lines, but also between the cell lines and hepatocytes. Figure 2.4 shows photographs of the panel of hepatoma cell lines. The Hep 3B2.1-7 cell line was epithelial-like and adhered well to the tissue culture plastic. Hep G2 cells also had an epithelial-like morphology, and contained only one nucleus (Wilkening et al, 2003). Huh-7D12 and PLC/PRF/5 were also epithelial-like and formed distinct colonies. SK-HEP-1 cells initially growed in aggregates around the few cells that manage to adhere to the plastic after which cells did grow in to bundles of spindle-shaped cells and formed a complete monolayer.

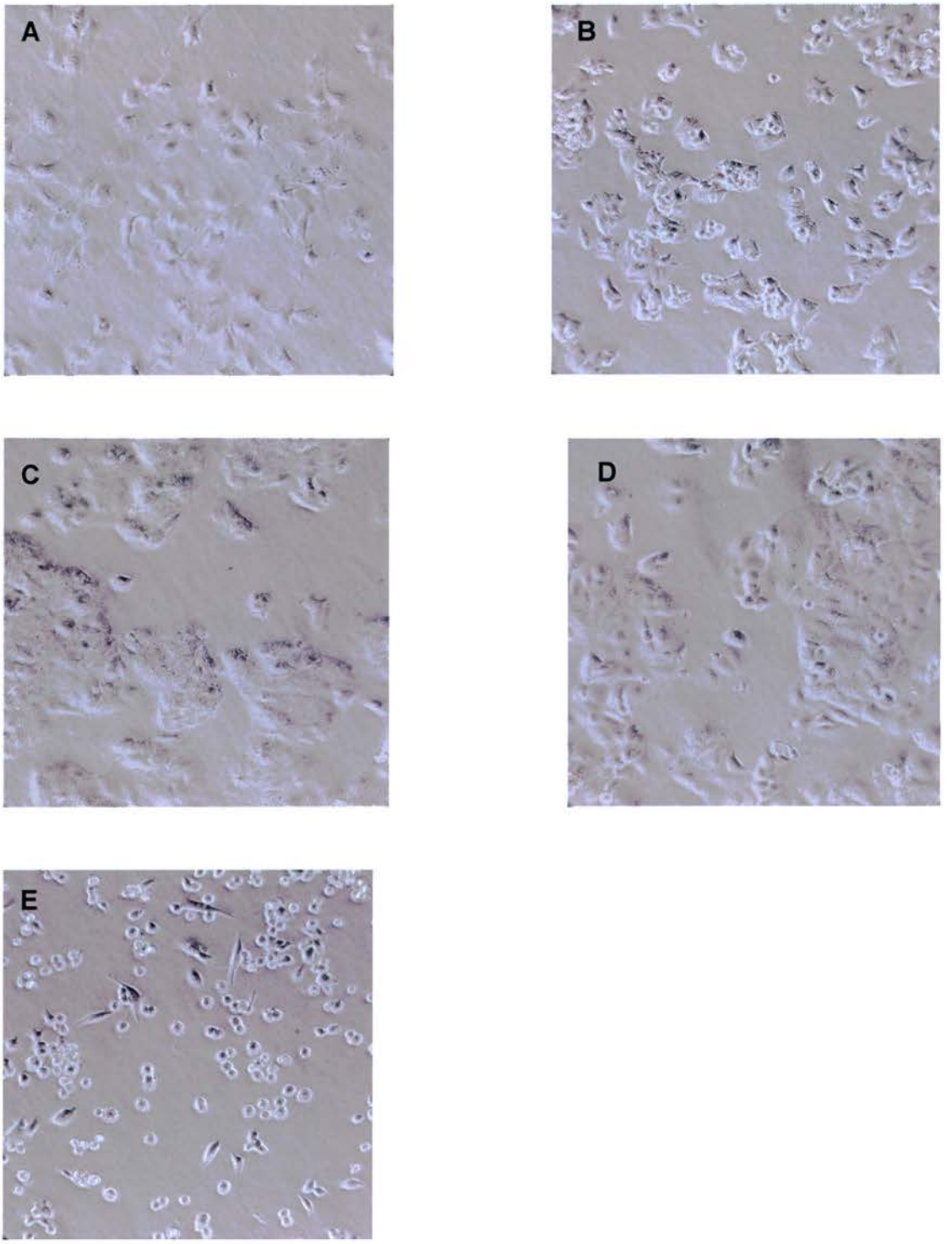


Figure 2.4: Morphological comparison of hepatoma cell lines in living monolayers

Cell lines were plated in supplemented DMEM for 24 hours before photographing. 1 x 10^6 Hep 3B2.1-7 (A), Hep G2 (B), Huh-7D12 (C), PLC/PRF/5 (D), and SK-HEP-1 (E) cells were plated per 25cm^2 petri dish and media removed before taking photographs using a Diavert microscope at x200 magnification.

2.3 VIABILITY OF HEPATOCYTES

During the procedure of isolating hepatocytes from liver, some of the cells suffer membrane damage and were therefore not viable. The process of cryopreservation reportedly further decreased the percentage of viable cells (Terry et al, 2005). The vital dye, trypan blue, is routinely used following hepatocyte isolation, and following the recovery of cells after cryopreservation, in order to determine the proportion of viable hepatocytes.

2.3.1 Fresh human hepatocytes

2.3.1.1 Initial viability and loss of viability during transport

The viability of isolated hepatocytes was determined immediately following isolation by the UKHTB, and at arrival in the laboratory in Edinburgh. Table 2.1 shows the percent cell viability after isolation, after transport, and the loss of viability during transport. The mean cell viability before and after transport was 82% and 67% respectively (medians were very similar to the means at 82% and 66% respectively), ranging from 70-92% initially, and 54-80% after transport. The median viability before and after transport were significantly different ($p<0.0001$) as determined by the non-parametric Wilkoxon matched pairs test.

Table 2.1: Fresh hepatocyte viability at isolation and after transport

SAMPLE I.D.	% CELL VIABILITY WHEN ISOLATED	% CELL VIABILITY AFTER TRANSPORT	% LOSS OF CELL VIABILITY
DONOR 1	83	65	18
DONOR 2	76	59	17
DONOR 3	70	54	16
DONOR 4	81	66	15
DONOR 5	79	60	19
DONOR 6	83	65	18
DONOR 7	85	77	8
DONOR 8	89	76	13
DONOR 9	80	62	18
DONOR 10	81	71	10
DONOR 11	82	67	15
DONOR 12	92	68	24
DONOR 13	82	80	2

The influence of donor factors on hepatocyte viability was assessed. Table 2.2 shows results of the statistical analysis performed. Donor age, sex or smoking status had no significant effect on hepatocyte viability both at isolation and after transport. Initial hepatocyte viability following isolation was found to be significantly correlated with hepatocyte viability after transport (Spearman correlation, r=0.7107, p=0.0065). However, the initial viability did not predict the extent of viability loss (Spearman correlation, r=-0.1021, p=0.74).

Table 2.2: Influence of donor demographics on cell viability
ns= non-significant (p>0.05)

	Donor Sex	Donor Age	Donor smoker/ non-smoker
Initial hepatocyte viability (%)	p=0.933 ns	r=0.146, p=0.6341 ns	p=0.199 ns
Hepatocyte viability after transport (%)	p=0.604 ns	r= 0.209 p=0.4936 ns	p=0.061 ns
Change in hepatocyte viability (%)	p=0.260 ns	r= -0.154 p=0.6153 ns	p=0.076 ns
Analysis performed	Mann Whitney test	Spearman correlation	Mann Whitney test

2.3.1.2 Viability during culture

The MTT assay, which is described in methods section 6.3.3, provides a measure of cell viability and was used for all of the cytotoxicity testing with fresh hepatocytes. OD values in control wells were monitored over the timecourse to be used in the cytotoxicity assays: 24 hours after the start of drug treatment (36 hours after plating), and 96 hours after the start of drug treatment (108 hours after plating). The MTT assay data suggest that the viability of hepatocytes from 9 out of 13 donors (69%) increased, and from 4 out of 13 donors decreased, over the 108-hour time period (figure 2.5). The MTT assay was used after plating of hepatocytes to detect viable cells, and has been recommended previously for the detection of hepatocyte viability in culture (Mitry et al, 2002). This method of measuring cell viability is more informative than using trypan blue as it measures cellular activity and requires functional mitochondria. When examining the MTT over time in culture, the OD

increased in hepatocytes from 9/13 donors and decreased in 4/13, over the time-course of the experiment. There are several possible explanations for this observation. The MTT assay measures a function of the mithochondria, therefore any change in the number or size of mitochondria in cells (Nagata, 2006) would alter the MTT conversion capability of the hepatocytes. The ability of cells to convert MTT over time in culture may also vary depending on the availability of required factors for metabolic conversion of MTT. The difference in attachment efficiency may explain the actual differences in OD values, but attachment efficiency was not measured in our experiments. For each experiment carried out using hepatocytes, each treatment had a corresponding set of control wells that were used to allow comparison of all the data, therefore the change in MTT conversion in control wells over time was taken in to account. Poor attachment to the tissue culture plastic could explain lower OD values obtained from the MTT assay, as number of functioning mitochondria will be lower the fewer the number of cells present. No significant correlation was found between initial hepatocyte viability or viability after transport and MTT OD either 36- or 108-hours after plating (Spearman correlation). The impact of stress gene expression on cell viability is considered in section 2.5.5.4.

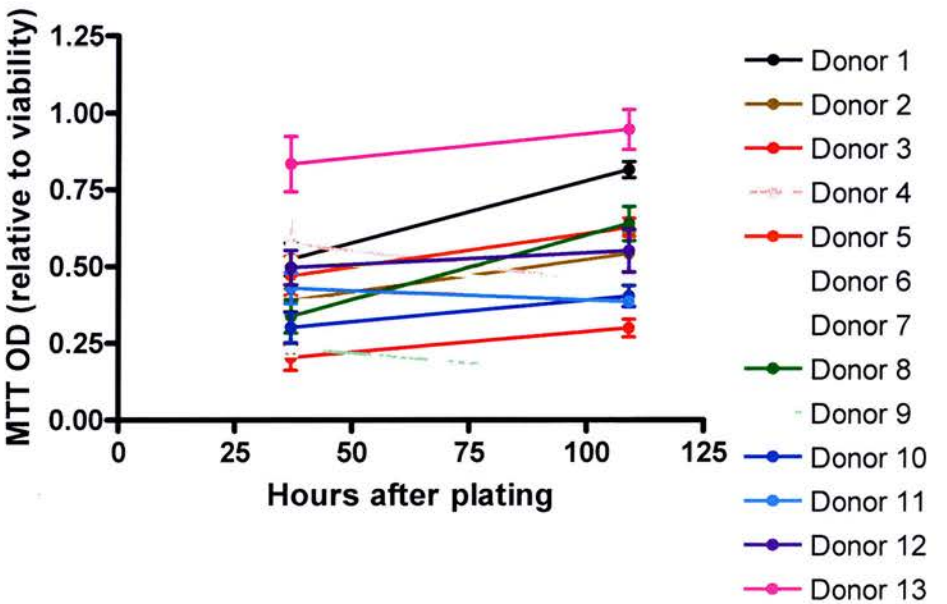


Figure 2.5: Cell viability determined by MTT assay over time in culture
20000 viable hepatocytes were plated per well and OD as determined by MTT assay was measured 36 and 108 hours after plating, as described in materials and methods.

2.3.2 Cryopreserved human hepatocytes

Cryopreserved hepatocytes from 4 donors were also obtained from the UKHTB and varied from donor to donor in terms of their viability and attachment. Viability at isolation ranged from 85-91% and at recovery after thawing from 41-59% (mean=49%) (See table 2.3), compared with 54-80% viability after transport in fresh hepatocytes. One of the main considerations for maintenance of hepatocyte-specific functions in culture, and ease of use for *in vitro* assays, is the attachment of cells to tissue culture plastic.

Hepatocytes from donors C2 and C4 were recovered from liquid nitrogen, and 6 hours later, good cell attachment to the plate was observed. The cells that were floating were removed and none of the floating fraction was viable as determined by trypan blue. The attached cells were used for cytotoxicity testing and RNA was also prepared from attached cells.

Sufficient attachment of hepatocytes from donor C3 (6 hours after recovery of cells from liquid nitrogen) allowed cytotoxicity testing. However, there were not enough cells available to extract RNA. Finally, hepatocytes from donor C1 when examined 24-hours after recovery from liquid nitrogen showed that cells had failed to attach to the tissue culture plastic. Trypan blue determination of cell viability revealed that none of the floating cells were viable.

Table 2.3: Viability of cryopreserved hepatocyte donors before and after cryopreservation

SAMPLE I.D.	% CELL VIABILITY WHEN ISOLATED	% CELL VIABILITY WHEN RECOVERED	% LOSS OF CELL VIABILITY
CRYO 1 (#5460)	91	49	42
CRYO 2 (#5514)	85	41	44
CRYO 3 (#5654)	89	47	42
CRYO 4 (#5354)	91	59	32

2.4 CELL GROWTH CHARACTERISTICS

An important factor to consider when selecting an *in vitro* model to represent the liver is the capability of the cells to proliferate. Whilst the liver does not proliferate in humans under normal conditions, the liver does have the ability to regenerate, for example after partial hepatectomy. The use of cell lines originating from the liver such as hepatoma cell lines to represent the fresh hepatocytes is based on the retention of some biochemical features of hepatocytes, and their capability of unlimited proliferation. There are technical advantages of using proliferating over non-proliferating cells for *in vitro* studies to represent the liver, and two different types of proliferating cell lines are considered here. For this study, the growth parameters evaluated were the doubling time, the plating efficiency (fraction of cells adhering to substratum), and the optimal plating density for 96-hour cytotoxicity assays in 96-well plates.

2.4.1 Hepatoma cell lines and immortalised hepatocytes

The doubling times for the five hepatoma cancer cell lines selected did not vary greatly from one another (table 2.4). Huh-7D12, HepG2 and SK-HEP-1 had similar doubling times (39-40 hours), whereas PLC/PRF/5 proliferated more rapidly (doubling time 33 hours) and Hep3B more slowly (54 hours). The immortalised hepatocyte cell line THLE-2 had a doubling time of 53 hours, and due to preliminary observations, a higher starting density of cells (20000 cells per well) was required for this replication rate otherwise the cells do not appear to proliferate. The plating efficiencies also vary (50-88% for cells lines and ~100% for THLE-2), as did the time to reach confluence, which resulted in different seeding densities for the cytotoxicity assays.

Table 2.4: Doubling time, plating efficiency & plating density per cell line (hepatomas, THLE-2)

CELL LINE	CELL DOUBLING TIME AT OPTIMUM CELL DENSITY (HOURS)	PLATING EFFICIENCY (%)	OPTIMUM PLATING DENSITY FOR CYTOTOXICITY STUDIES (cells/well)
Huh-7 D12	40	88	5000
HepG2	39	71	5000
Sk-Hep-1	40	50	5000
Hep3B	54	77	5000
PLC/PRF/5	33	73	3000
THLE-2	53	~100	20000

2.5 GENE EXPRESSION OF *IN VITRO* MODELS

The expression of genes with particular relevance to the liver (see table 2.5) was assessed by qRT-PCR. Several liver-specific genes were selected to compare the gene expression profile of the different cell lines to fresh hepatocytes. Alpha fetoprotein (AFP) was also evaluated as it can be used as a marker in humans for the detection of hepatocellular carcinoma and therefore is likely to be expressed in hepatoma cell lines. In order to evaluate the level of stress induced in the hepatocytes due to transport and plating, HIF1α, HSP70 and p21 gene expression was determined as these are all genes that may be upregulated in response to stress stimuli. Gene expression was determined in all of the liver models considered in these studies (fresh, cryopreserved and immortalised hepatocytes and hepatoma cell lines). mRNA expression was detected by qRT-PCR in fresh hepatocytes from 13 donors, cryopreserved hepatocytes from 2 donors, 5 hepatoma cell lines and the immortalised hepatocyte cell line, THLE-2.

Table 2.5: Genes of interest, reference genes, and references for information on each gene

Type of gene	Gene (Genecard name)	References
Liver-expressed	Albumin (ALB)	Farrell, 1994.
	α-1-antitrypsin (AAT)	Perlmutter, 2002.
	CYP1A1	Binda et al., 2003, Martignoni et al., 2006, Lekas et al., 2002.
	CYP3A4	Binda et al., 2003, Martignoni et al., 2006,Guengerich et al., 1999, Nishimura et al., 2005, Raucy, 2003, Pascussi et al., 2002, Thummel et al., 1998, Usui et al., 2003.
	Transthyretin (TTR)	Blaner et al., 1991.
	UDP-glucuronosyltransferase (UGT1A* and UGT2B*)	King et al., 2000.
	Nuclear receptor subfamily 1, group I, member 2 (Pregnane X receptor) (PXR)	Handschin and Meyer, 2003.
	ATP-Binding Cassette, sub-family B (MDR/TAP), member 1 (ABCB1)	DeLeve, 2003.
Foetal liver-specific	Alpha-fetoprotein (AFP)	Matsumura et al., 2001.
Stress genes	HIF1α	Minchenko et al., 2002, Sonna et al., 2003.
	HSP70	Joo et al., 2005, Kregel et al., 2002, Sonna et al., 2002, Tokyol et al., 2005, Yamamoto et al., 1998.
	p21	Crary et al., 1998, Kwon et al., 2003, Borozan et al., 2006.
Reference genes	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Blanquicett et al., 2002, Macpherson et al., 2006, Radonic et al., 2004, Schmittgen et al., 2000, Vandesompele et al., 2002, Bustin, 2000.
	18S rRNA	
	β2-Microglobulin (β2m)	

2.5.1 Quantitative RT-PCR set-up

One of the critical requirements in order to produce reliable and reproducible qRT-PCR data is the RNA quality. Therefore, the quality of the RNA extracted from the hepatocytes was verified by miniaturised electrophoresis using an Agilent Bioanalyser with the RNA 6000 Nano Assay (see chapter 6). All hepatocyte RNA samples had RNA integrity numbers (RIN) between 6.7 and 9.3, with the exception of one sample from donor 10 (H24d) (RIN = 2.5), which was excluded from the analysis of gene expression.

Another issue to be considered for qRT-PCR is the design and optimisation of suitable primers. The primers were designed using primer3 software (see chapter 6). Optimisation was carried out using the melting curve of the PCR products to ensure a single product (Figure 2.6 A), and product size was verified by separating products by agarose gel electrophoresis (see chapter 6).

Standard curves were established using a commercial supply of adult human liver RNA for all genes of interest, except AFP for which commercially available pooled foetal liver RNA was used. A standard curve was generated from the cycling data during the exponential phase of the PCR reaction (see figure 2.6 B) and used to determine gene expression in samples. The range of linearity of the standard curves and the efficiency of the amplification were verified for each primer set (see table 2.6)

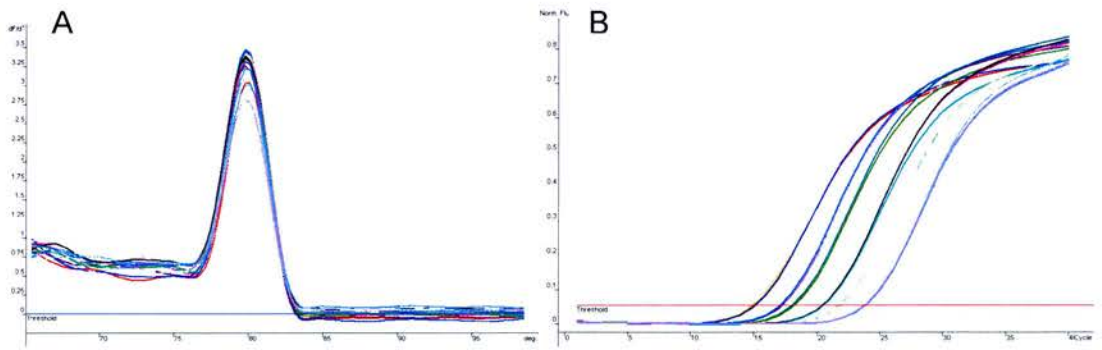


Figure 2.6: CYP3A4 product melt curve (A) and rotorgene cycling (B)

Table 2.6: Standard curve parameters and melting temperature of qRT-PCR products

GENE (abbreviation)	RNA standard curve range (ng)	Reaction efficiency	r^2	Melting Temperature (°C)
ALB	BD adult liver RNA 50-0.05	0.95-1.17	0.979-0.999	78.8-79.7
AAT	BD adult liver RNA 50-0.05	1.04-1.11	0.983-0.994	84.5-85.5
CYP1A1	BD adult liver RNA 10-0.1	0.94-1.13	0.990-0.997	82.7-83.0
CYP3A4	BD adult liver RNA 50-0.1	1.01-1.07	0.983-0.998	79.2-80.0
TTR	BD adult liver RNA 50-0.1	0.99-1.18	0.983-0.992	83.3-84.0
UGT1A*	BD adult liver RNA 50-0.1	1.01-1.15	0.991-0.996	84.2-85.2
UGT2B*	BD adult liver RNA 50-0.1	1-1.06	0.974-0.997	80.3-81.5
ABCB1	BD adult liver RNA 50-1	1.1-1.17	0.988-0.989	81.5-82.0
PXR	BD adult liver RNA 50-0.1	1.06-1.24	0.991-0.997	83.5-84.3
AFP	BD foetal liver RNA 10-0.01	0.95-1.11	0.993-0.998	81-81.8
HIF1α	BD adult liver RNA 50-1	1.14-1.2	0.988-0.991	78.3
HSP70	BD adult liver RNA 50-0.5	0.96-1.13	0.982-0.994	88.3
p21	BD adult liver RNA 10-0.1	1-1.02	0.986-0.991	83.8
GAPDH	BD adult liver RNA 50-0.1	1.07-1.21	0.992-0.998	84.2-85.3
β_2M	BD adult liver RNA 50-0.1	0.85-0.97	0.992-0.999	77.8-78.3
18S	BD adult liver RNA 1-0.01	0.75-0.84	0.995-0.997	81-81.8

2.5.2 Reference Gene Selection

Accurate determination and quantification of gene expression requires normalisation of the results using the expression of an ubiquitously expressed internal standard, reference gene, or housekeeping gene (HKG) (Blanquicett et al., 2002; Bustin and Nolan, 2004) (see table 2.5). The accurate quantification of a true reference gene allows the normalisation of differences in the amount of amplifiable RNA or cDNA generated in different samples by different amounts of starting material; the quality of the starting material, and; differences in RNA preparation and cDNA synthesis, since the reference gene is exposed to the same preparation steps as the gene of interest (Radonic et al., 2004). However, several studies (e.g. Blanquicett et al., 2002) have suggested that many HKGs demonstrate variability between different tissues and/or disease states. An ideal reference gene would be constantly transcribed in all cells and tissues, and its RNA transcription level would not be altered by internal or external influences. When dealing with patient samples, it is recommended to evaluate several reference genes and to normalise the results by the geometric mean of the most appropriate ones (Vandesompele et al., 2002). Significant variation in the expression of reference genes between carcinomatous and normal liver samples has been reported (Blanquicett et al., 2002) and has implications for the studies presented here. Therefore selection and validation of possible reference genes was undertaken.

Three reference genes were tested: 18S rRNA, β_2-microglobulin (β_2-M) mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. 18S is ribosomal RNA (rRNA) and together with 28S makes up 80% of total RNA, is transcribed by a different polymerase from mRNAs, and its level is less likely to fluctuate with the test sample (Huggett et al, 2005). β_2-M is involved in the immune response, and is the β chain of major histocompatibility complex class I molecules. GAPDH is a glycolytic enzyme that is widely cited and commonly used as a housekeeping gene (Huggett et al, 2005).

B_2M, GAPDH and 18S mRNA expression was determined in hepatocyte samples from donors 1-11. The resulting expression was analysed using GeNorm software

(Vandesompele et al., 2002), and a 'normalisation factor' was generated using the B_2M, GAPDH and 18S mRNA expression data from hepatocyte donors 1 to 11. Based on the GeNorm analysis B_2M and GAPDH mRNA expression was found to be less variable than 18rRNA expression between samples (figure 2.7), therefore the geometric mean of GAPDH and B_2M was also calculated. The gene or combination of genes that shows least variability between samples should be selected to normalise the gene expression data for the genes of interest. For practical considerations and accuracy the geometric mean of the 2 reference genes with least variability between samples (GAPDH and B_2M), was calculated for each sample and used to normalise the gene expression data (see figure 2.7). It should also be noted that the geometric mean is better than the arithmetic mean for this purpose because the former controls better for outlying values and abundance differences between the different genes.

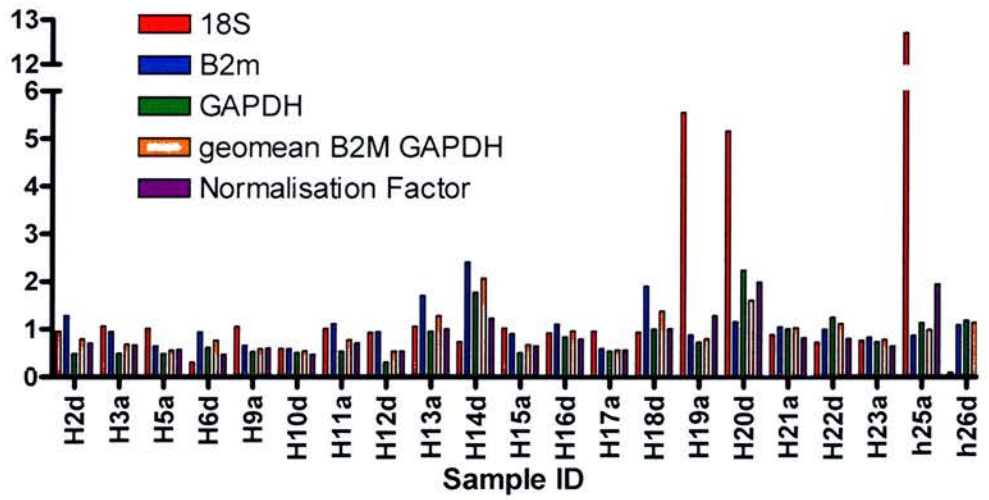


Figure 2.7: Comparison of reference genes expression in hepatocytes
18S, B_2M, and GAPDH mRNA expression was determined by qRT-PCR in hepatocytes from donors 1-11. The geometric mean of B_2M and GAPDH, and the normalization factor as determined by Genorm software is also plotted.

2.5.3 Expression of liver-specific genes in fresh hepatocytes

The expression of a selection of liver-specific genes (see table 2.5) was determined in fresh hepatocytes from 13 donors at two time-points: after transport and arrival in Edinburgh, and 12 hours after arrival, following plating in collagen-I pre-coated tissue culture flasks. The mean gene expression decreased from after transport to after plating for all investigated genes except CYP1A1, the expression of which was maintained, and ABCB1 the expression of which increased following plating. For all genes except CYP1A1, hepatocytes from the majority of donors had expression >1 when compared to the standard liver RNA that was used for relative quantification. Possible reasons for this are discussed in section 2.7. For several of the investigated genes in hepatocytes from the majority of donors, expression was maintained >1 after plating and therefore at the start of drug treatment. The examination of individual samples revealed that the expression of all genes except ABCB1 decreased in expression between arrival and overnight plating in hepatocytes from the majority of donors.

Normalised α-1-antitrypsin mRNA expression (see figure 2.8) ranged from 0.63 to 5.10 in fresh human hepatocytes before plating, and from 0.39 to 2.10 12-hours following plating. For all donors (excluding donors 4 and 8 in which expression increased, and donor 10 for which sample H24d was not analysed) AAT mRNA expression decreased between the two time-points. In hepatocytes from 50% of donors, albumin mRNA expression was >1 at the start of drug treatment and therefore at a higher level than in the commercially available reference RNA.

Normalised albumin mRNA expression ranged from 0.63-5.6 in fresh human hepatocytes before plating, and from 0.22-2.6 12-hours following plating and transthyretin ranged from 0.19 to 3.3 before plating, and from 0.08 to 2.5 12-hours following plating (see figure 2.8). For all donors (excluding donor 10 for which sample H24d was not analysed) albumin and TTR mRNA expression decreased between the two time-points. However, it should be noted that for 7 out of 12 donors albumin mRNA expression was >1 at the start of drug treatment and therefore at a

higher level than in the commercially available reference RNA. Conversely, for 7 out of 12 donors TTR mRNA expression was <1 at the start of drug treatment.

Normalised CYP3A4 mRNA expression ranged from 0.02 to 12 in fresh human hepatocytes before plating, and from 0.24 to 4.4 12-hours following plating, with CYP3A4 not being detectable in hepatocytes from donor 5 12-hours following plating (see figure 2.8). Normalised PXR mRNA expression ranged from 1.7 to 8.3 in fresh human hepatocytes before plating, and from 0.95 to 3.6 12-hours following plating (see figure 2.8). For all donors (excluding donor 10 for which sample H24d was not analysed) CYP3A4 and PXR mRNA expression decreased between the two time-points. For 8 out of 12 donors CYP3A4 and PXR mRNA expression >1 at the start of drug treatment and therefore at a higher level than in the commercially available reference RNA.

The expression of the UGT2A* and UGT2B* subfamilies of UDP-GTs was also analysed by qRT-PCR (see figure 2.8). Normalised UGT1A* mRNA expression ranged from 0.50 to 3.9 in fresh human hepatocytes before plating, and from 0.15-2.0 12-hours following plating. Normalised UGT2B* mRNA expression ranged from 0.78 to 3.70 before plating, and from 0.21-2.50 12-hours following plating. For all donors (excluding donor 8 which increased, and donor 10 for which sample H24d was not analysed) UGT1A* and UGT2B* mRNA expression decreased between the two time-points. For 7 and 9 out of 12 donors for UGT1A* and UGT2B* respectively, mRNA expression was <1 at the start of drug treatment and therefore at a lower level than in the commercially available reference RNA.

Normalised CYP1A1 mRNA expression (see figure 2.8) ranged from 0.14 to 3.03 in fresh human hepatocytes before plating, and from 0.03 to 5.60 12-hours following plating. For all donors (excluding donors 2, 5 and 13 in which expression increased, and donor 10 for which sample H24d was not analysed) CYP1A1 mRNA expression decreased between the two time-points. For 10 out of 12 donors CYP1A1 mRNA expression was <1 at the start of drug treatment and therefore at a lower level than in the commercially available reference RNA.

The only gene whose expression increased following plating in all donors (excluding donor 10 for which sample H24d was not analysed) was ABCB1. Normalised ABCB1 mRNA expression (see figure 2.8) ranged from 0.43 to 5.40 in fresh human hepatocytes before plating, and from 4.0 to 18.0 12-hours following plating. For all donors ABCB1 mRNA expression was >1 at the start of drug treatment and therefore at a higher level than in the commercially available reference RNA.

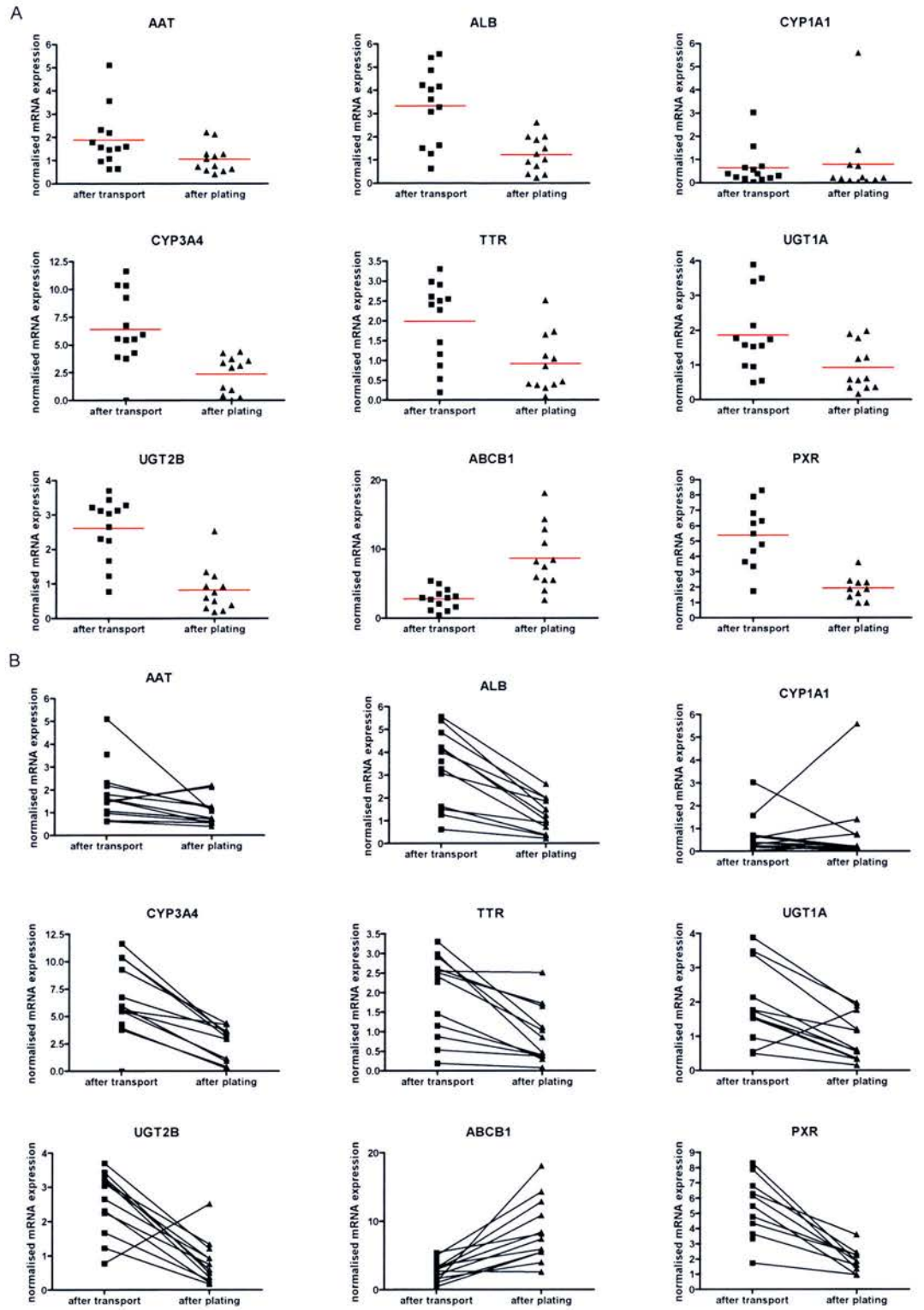


Figure 2.8: Gene expression in fresh human hepatocytes
mRNA expression of liver-specific genes as determined by qRT-PCR in fresh human hepatocytes from donors 1-13 before and 12-hours after plating. Results are expressed as normalized gene expression per time point per donor. A) demonstrates the interindividual variability and the red line represents the mean of 13 donors. B) demonstrates before and after plating gene expression data for each donor.

2.5.4 Expression of liver-specific genes in cryopreserved hepatocytes

The gene expression analysis was only possible in cryopreserved hepatocytes from two donors, due to the limited number of cryopreserved hepatocytes available for RNA extraction. The results are presented in figure 2.9 and are summarised in table 2.7 below. In summary cryopreserved hepatocytes from donors C2 and C4 expressed detectable levels of all the liver-specific genes investigated (except AFP, as expected). ABCB1, CYP3A4, UGT1A* and UGT2B* mRNA expression levels in both cryopreserved donors were comparable with mRNA expression in the fresh hepatocytes.

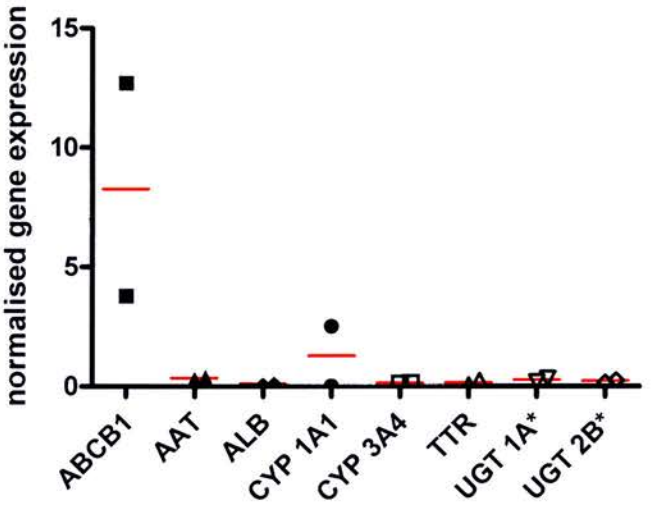


Figure 2.9: Gene expression in cryopreserved human hepatocytes
mRNA expression was determined by qRT-PCR in hepatocytes from donors C2 and C4 and normalised to the geometric mean of B_2M and GAPDH. The red line represents the mean normalised mRNA expression from the 2 donors.

Table 2.7: Gene expression in cryopreserved hepatocytes
Results presented are normalised mRNA expression and are calculated as mean of triplicates.

Donor/ cell line	ABC B1	AAT	AFP	ALB	CYP 1A1	CYP 3A4	TTR	UGT 1A*	UGT 2B*
C2	13	0.29	nd	0.06	0.02	0.12	0.05	0.18	0.17
C4	3.8	0.37	nd	0.10	2.5	0.14	0.24	0.33	0.26

2.5.5 Expression of liver-specific genes in immortalised and hepatoma cell lines

The liver-specific genes that were assessed in the fresh and cryopreserved hepatocytes were also investigated in 5 hepatoma cell lines and in THLE-2 cells. An additional gene was also investigated, namely alpha-fetoprotein, in the cell lines. AFP is produced during human embryonic development by the foetus and is detectable in the serum of pregnant women because it crosses the placental barrier. Levels are undetectable after a year of age in humans and AFP is not detectable in healthy adults. AFP expression can increase in the presence of hepatocellular carcinoma or germ cell tumours and is used as a marker in patients these diseases. High AFP levels suggest the presence of liver cancer, and AFP may be expressed in hepatoma cell lines. AFP expression was assessed in all of the models investigated in this project. Four of the hepatoma cell lines (HepG2, Hep3B, PLC/PRF/5 and Huh-7D12) expressed AFP, with HepG2 and Huh-7D12 having the highest levels of expression, as can be seen in figure 2.10. AFP expression was not detectable in any of the hepatocytes or in the THLE-2 cell line.

Generally expression levels were lower in the hepatoma cell lines than had been identified in the fresh hepatocytes. Albumin mRNA expression was not detectable in PLC/PRF/5 or SK-HEP-1 and ranged from 0.02 (relative to HKG) in Huh-7D12 to 0.19 in Hep G2 cells. AAT was expressed at a low level (0.02) in THLE-2 cells, and in the hepatoma cell lines mRNA expression was not detectable in SK-HEP-1 and ranged from 0.05 in Huh-7D12 to 0.58 in Hep G2 cells. CYP1A1 was expressed at a low level (0.03) in THLE-2 cells, and in the hepatoma cell lines mRNA expression was not detectable in SK-HEP-1 and ranged from 0.01 in Hep G2 to 0.45 in PLC/PRF/5 cells. CYP3A4 was not detectable in THLE-2 cells, or in any of the hepatoma cell lines except for Hep 3B2.1-7 cells (0.01). TTR mRNA expression was not detectable in SK-HEP-1, PLC/PRF/5 or Hep 3B2.1-7 cells and was expressed at low levels in Huh-7D12 and HepG2 (0.01 and 0.04 respectively). UGT1A* and PXR mRNA expression was not detectable except in Hep G2 cells (0.01 and 2.60 respectively). UGT2B* mRNA expression ranged from undetectable in SK-HEP-1 and PLC/PRF/5 to 0.35 in Hep 3B2.1-7 cells. ABCB1 mRNA

expression was not detectable in SK-HEP-1 or PLC/PRF/5 and ranged from 0.30 in HepG2 to 0.60 in Hep 3B2.1-7 cells. ALB, CY3A4, TTR, UGT1A*, UGT2B*, PXR and ABCB1 were not detectable in THLE-2 cells.

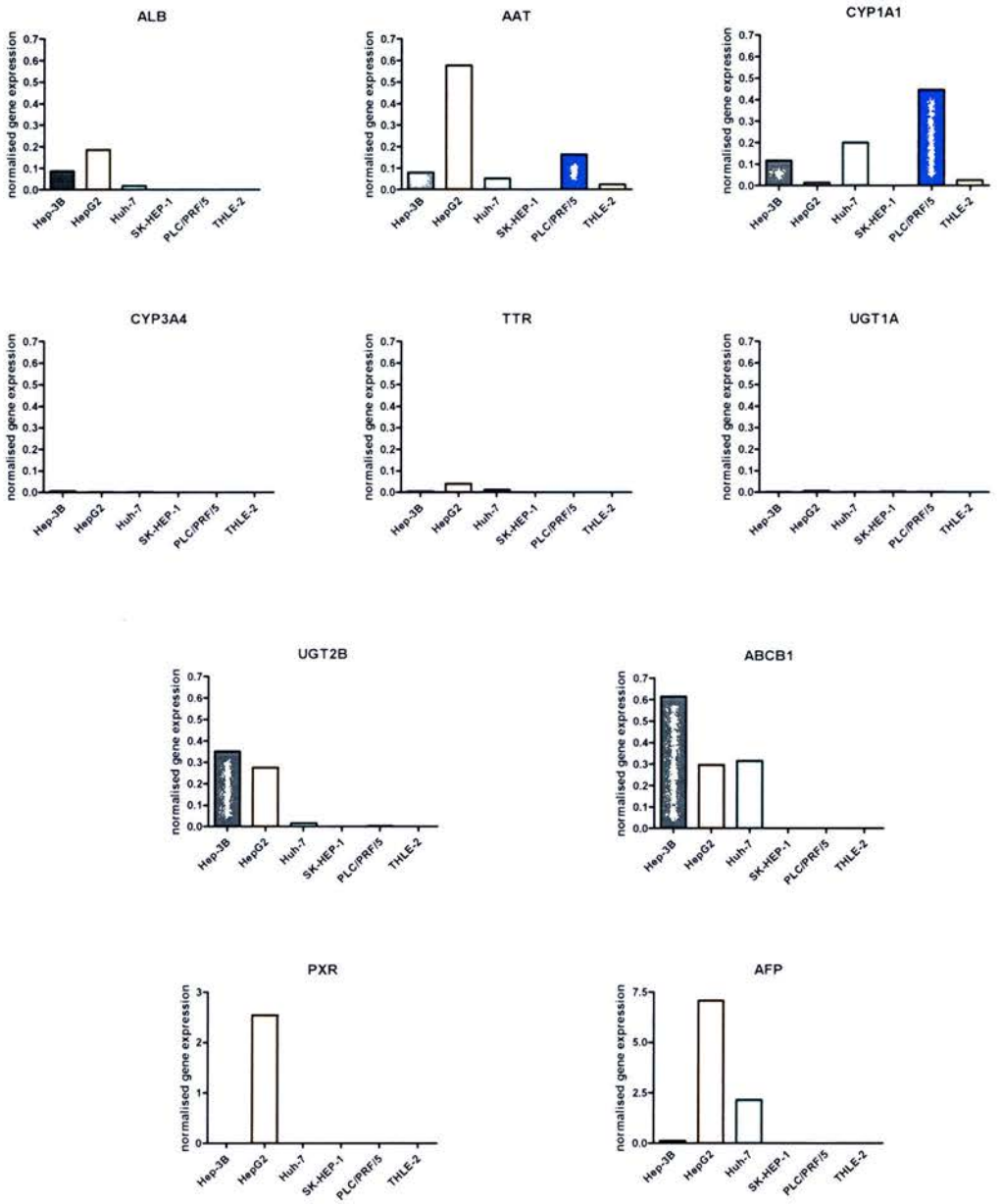


Figure 2.10: Gene expression in 5 hepatoma cell lines and immortalised hepatocyte cell line THLE-2

mRNA expression was determined by qRT-PCR in 5 hepatoma cell lines and THLE-2 and normalised to the geometric mean of B_2M and GAPDH.

2.5.6 Comparison of gene expression between *in vitro* models

A selection of 10 genes whose expression is particularly relevant to the liver has been investigated. There was marked variability in mRNA expression of each gene between the models and the range of normalised gene expression is presented in figure 2.11. Fresh hepatocytes from all 13 donors expressed all of the liver-specific genes investigated (except AFP, as expected). In general, for the genes investigated, mRNA expression was higher in fresh hepatocytes from all donors than in any of the other models investigated.

With regards to the fresh hepatocytes, there is inter-donor variability for all genes both before plating (after transport) and after plating (at the start of drug treatment) (figure 2.11). For the liver-specific genes investigated, the mean expression from all fresh hepatocyte donors is higher immediately after transport than after plating, with the exception of CYP1A1 and ABCB1. Gene expression in the cryopreserved hepatocytes is less than in fresh hepatocytes both before and after plating and lower than that in the reference RNA for all genes except ABCB1 and CYP1A1. In all the hepatoma cell lines investigated, expression of all genes was lower than the mean expression in fresh hepatocytes (before and after hepatocyte transport) and lower than in the reference RNA. Expression of the investigated genes varied widely between the hepatoma cell lines. AFP expression was detected in the hepatoma cell lines, and the highest expressers were HepG2 and Huh-7D12 when compared with all the other models. HepG2 expressed all genes except CYP3A4, but only AAT and UGT2B* mRNA expression levels were comparable with those of fresh hepatocytes, with all of the other genes being expressed at a lower level. Huh-7D12 expressed all of the liver-specific genes investigated, with the exception of CYP3A4 and UGT1A*. For all genes except CYP1A1, mRNA expression was lower than in the fresh and cryopreserved hepatocytes from all donors. Hep3B2.1-7 expressed all liver-specific genes except TTR and UGT1A*, and for all genes the expression was lower than that in the majority of fresh hepatocytes. PLC/PRF/5 had only very low expression of AAT, AFP and CYP1A1, and SK-HEP-1 expressed none of the investigated genes. The only genes that were detectable in the THLE-2 cell line were

AAT and CYP1A1, and mRNA expression was extremely low (0.02 and 0.03 respectively).

In summary, the fresh hepatocytes recapitulate the expected expression of the selected genes after plating better than any of the other models investigated. Therefore if a decision was to be made as to the most appropriate *in vitro* model to represent the liver solely based on the gene expression analysis, fresh hepatocytes would be selected. There is variation in expression of all genes between donors and this is representative of the situation in humans where inter-individual variation in gene expression occurs.

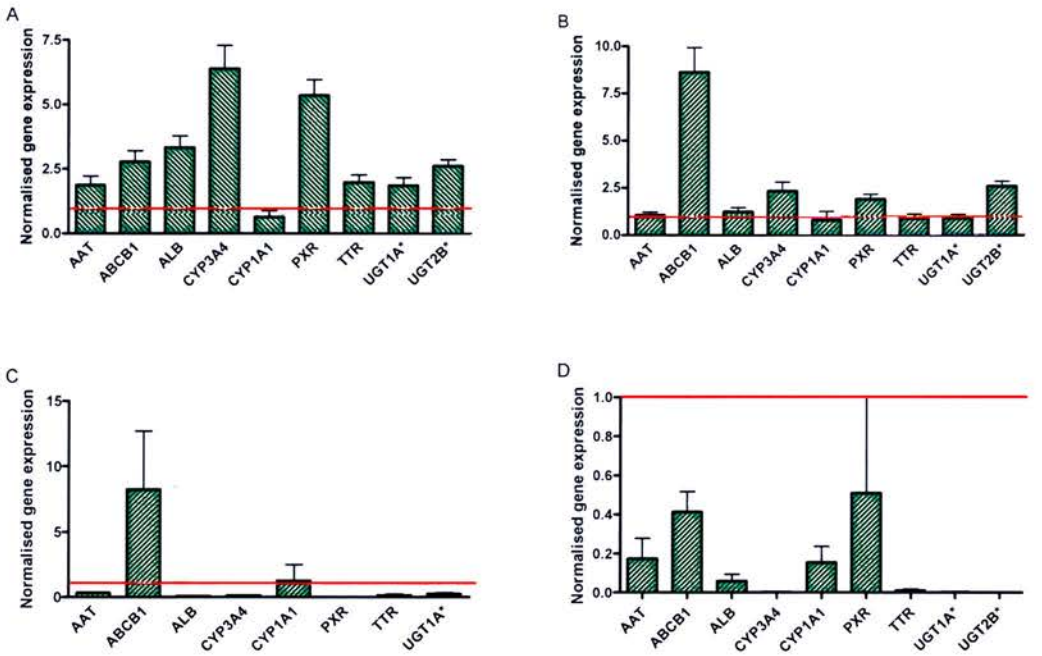


Figure 2.11: Gene expression in hepatocytes before and after plating, cryopreserved hepatocytes and hepatoma cell lines
mRNA expression of liver-specific genes as determined by qRT-PCR in fresh human hepatocytes from donors 1-13 before (A) and 12-hours after (B) plating, cryopreserved hepatocytes from 2 donors (C), and in 5 hepatoma cell lines (D). Results are expressed as mean and standard deviation of normalised gene expression. The red line indicates a normalised gene expression value of 1, equivalent to the expression in the liver standard RNA.

2.5.7 Expression of stress-induced genes in hepatocytes

During liver resection, the cells are subjected to several stress stimuli, including innate and antigen-independent stresses. These may include the hepatic response to systemic cytokines activated in response to the stress of surgery or brain death, and hypothermia and preservation injury of cold ischaemia (Borozan et al, 2006). Furthermore, the isolation of hepatocytes from the liver with collagenase may result in stress response and hypoxia. In order to determine the extent of stress response induced in the fresh hepatocytes used in these studies, the expression of hypoxia-inducible factor 1, alpha subunit (HIF1A), heatshock protein 70kDa protein 1A (HSP70) and Cyclin-dependent kinase inhibitor 1A (CDKN1A, p21, Cip1) was determined.

p21 is involved in cell cycle regulation and is a target gene for p53 during apoptosis (Dotto, 2000). A study by Borozan et al. (2006) demonstrated that p21 was upregulated during liver resection, compared to when the liver was first exposed and confirmed this finding by both RT-PCR and microarray techniques. The authors suggest that p21 may have an important role in regulating hepatocyte cell cycle progression in liver regeneration (Crary et al, 1998). Heat shock proteins are a group of proteins whose expression may be increased in response to elevated temperature and other 'stress' conditions, and Borozan et al (2006) also demonstrated that HSP-70 was upregulated in response to acute hepatic stress. The isolation of hepatocytes from the liver with collagenase may also result in hypoxia. HIF1A functions as a master transcriptional regulator of the adaptive response to hypoxia, and under hypoxic conditions activates the transcription of more than 60 genes (Lee et al, 2004).

2.5.7.1 HIF1A, p21 and HSP-70 mRNA expression in fresh hepatocytes

In all pairs of hepatocytes samples, HIF1A expression increased between arrival and 12-hours after plating (see figure 2.12A), with mRNA expression ranging from 3.5 to 7.3 after transport, and from 13.8 to 44.5 after plating. Similarly, in all pairs of hepatocyte samples with the exception of donor 12, p21 expression increased

between arrival and 12-hours after plating (see figure 2.13A), with mRNA expression ranging from 3.15 to 11.76 after transport, and from 6.48 to 14.87 after plating. Finally, heat shock 70kDa protein (HSP70) expression increased in all pairs of hepatocytes samples from initially after transport to 12-hours after plating (see figure 2.14A), from 0.27 to 1.11 after transport, to 0.38 to 12.72 after plating. The difference in gene expression after transport and after plating for all genes was found to be statistically significant with a non-parametric Wilcoxon matched pairs test ($p = 0.0005$, 0.0093 and 0.0005 for HIF1A, p21 and HSP-70 respectively). In all cases, the gene expression at arrival did not correlate with gene expression after plating (figures 2.12B, 2.13B and 2.14B).

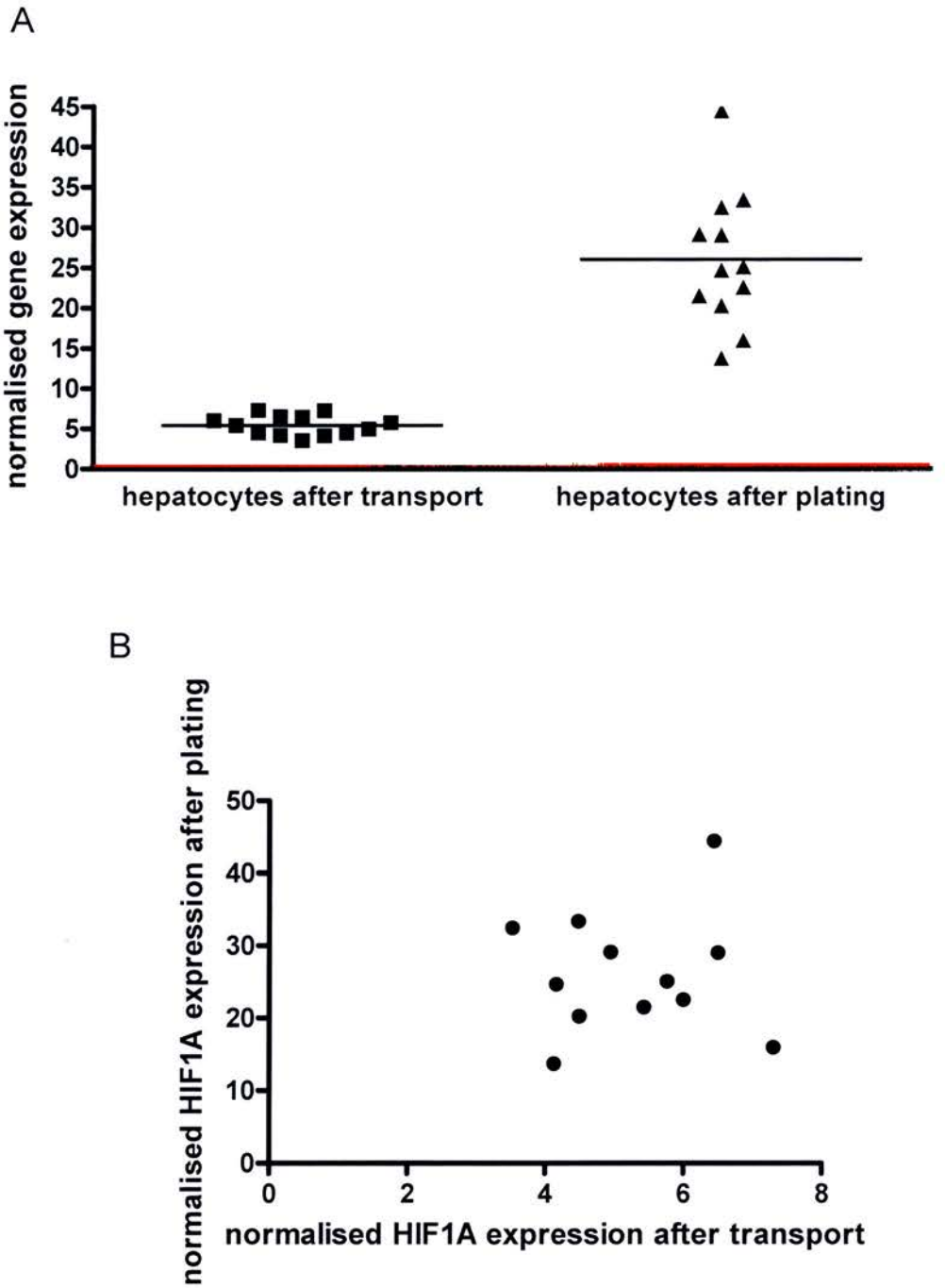


Figure 2.12: HIF1A mRNA expression in fresh hepatocyte samples
Gene expression determined after qRT-PCR analysis. HIF1A expression was determined following arrival of the hepatocytes in Edinburgh, and after plating for 12 hours (A). Results are normalised to the geometric mean of GAPDH and β_2M, and are mean of triplicates. The relationship between normalised HIF1A expression after transport, and normalised HIF1A expression after plating was also investigated (B).

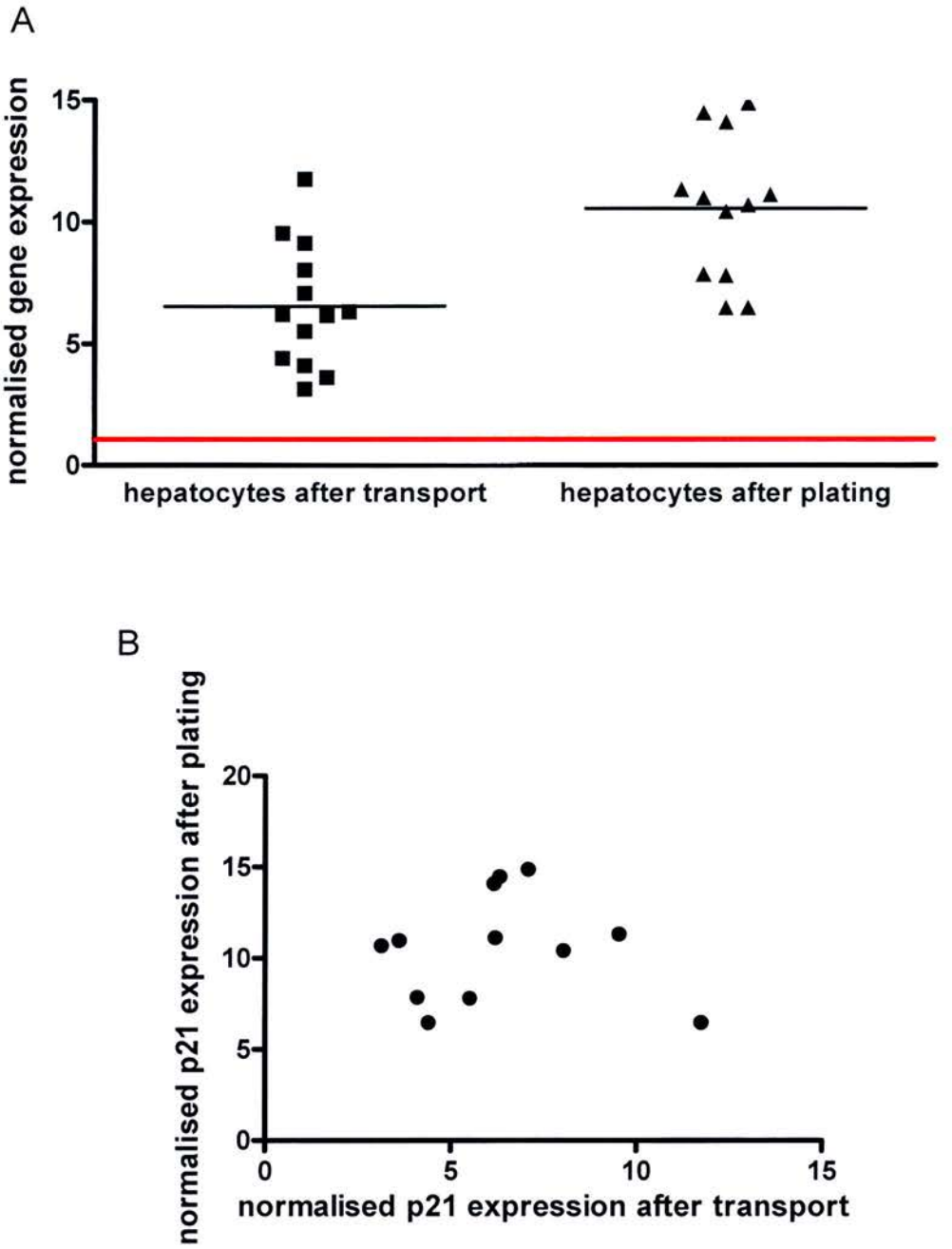


Figure 2.13: p21 mRNA expression in fresh hepatocyte samples
Gene expression determined after qRT-PCR analysis. p21 expression was determined following arrival of the hepatocytes in Edinburgh, and after plating for 12 hours (A). Results are normalised to the geometric mean of GAPDH and β_2M, and are mean of triplicates. The relationship between normalised p21 expression after transport, and normalised p21 expression after plating was also investigated (B).

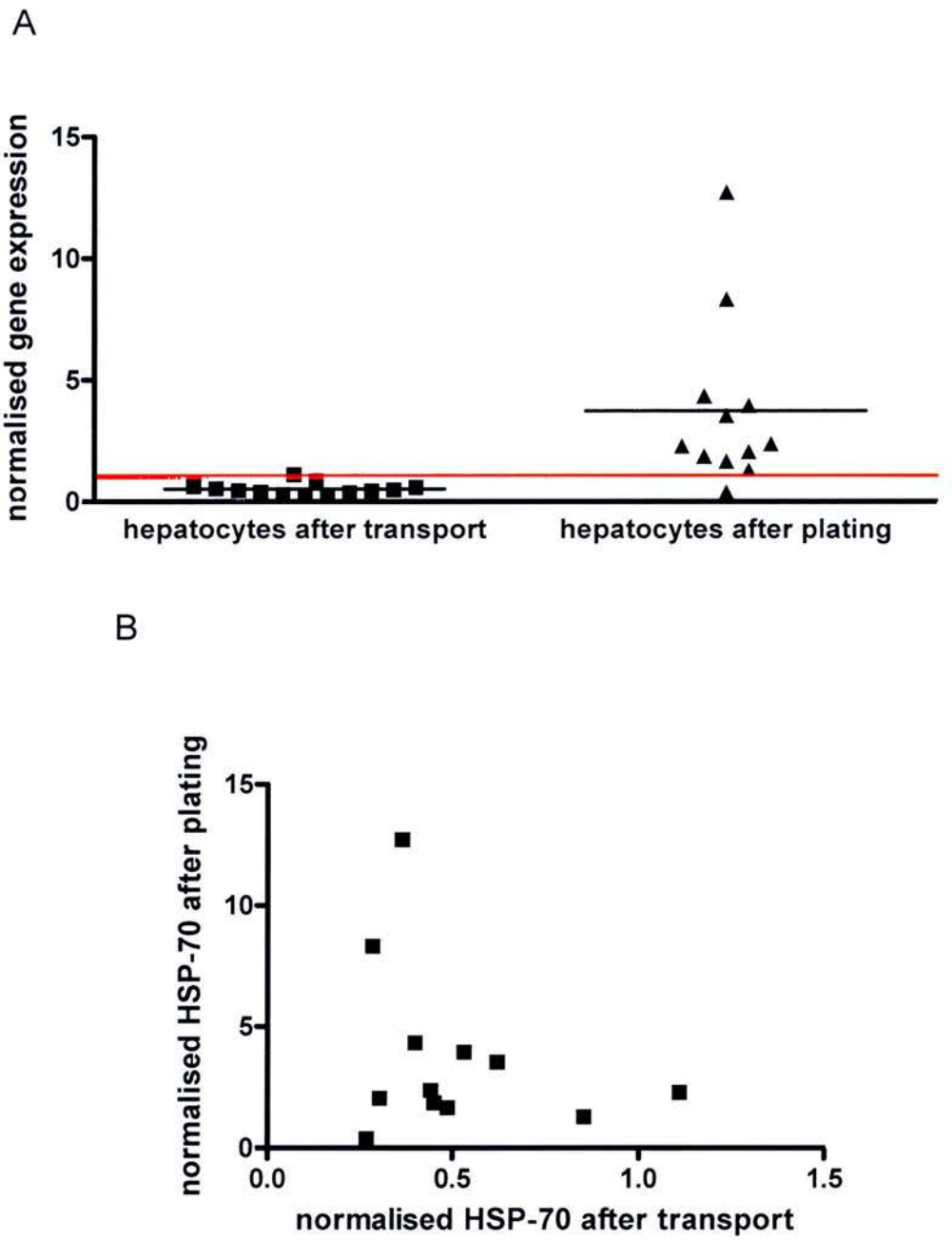


Figure 2.14: HSP-70 mRNA expression in fresh hepatocyte samples
Gene expression determined after qRT-PCR analysis. HSP-70 expression was determined following arrival of the hepatocytes in Edinburgh, and after plating for 12 hours (A). Results are normalised to the geometric mean of GAPDH and β_2M, and are mean of triplicates. The relationship between normalised HSP70 expression after transport, and normalised HSP70 expression after plating was also investigated (B).

2.5.7.2 Stress-induced genes in cryopreserved hepatocytes

The expression of p21, HSP-70 and HIF1A was also determined in cryopreserved hepatocytes. Normalised HIF1A and HSP-70 expression levels were similar in cryopreserved hepatocytes from both donors, however p21 expression was higher in donor C4 than in C2 (17.5 compared to 4.3) (see figure 2.15). Similar to the fresh hepatocytes, mRNA expression of all stress genes investigated was higher in these samples than in the RNA standard and also higher than the expression of liver-specific genes. HIF1A and p21 expression was within the same range of that in fresh hepatocytes after plating, however the mean HSP-70 expression was higher in the cryopreserved than in fresh hepatocytes.

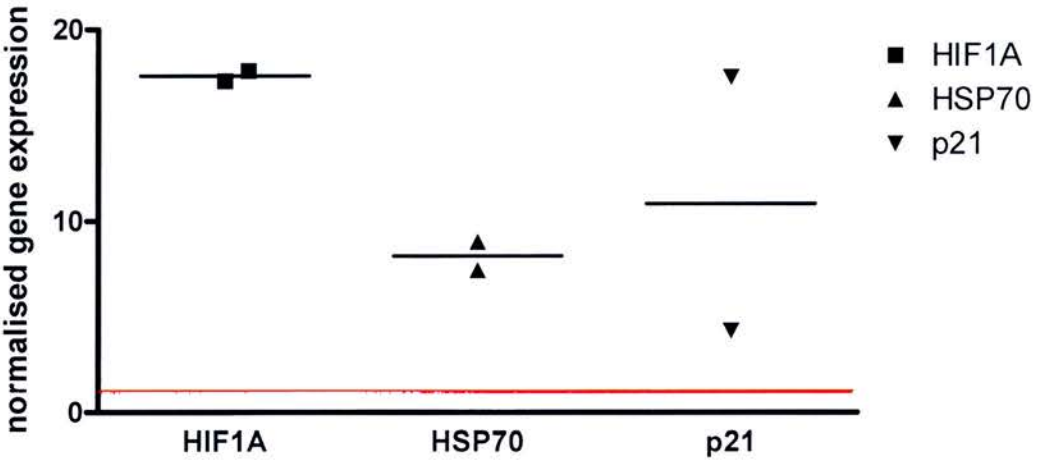


Figure 2.15: HIF1A, HSP70 and p21 expression in cryopreserved human hepatocytes

Gene expression determined after qRT-PCR analysis of stress in genes in cryopreserved hepatocytes from donor C2 and C4. Results are normalised to the geometric mean of GAPDH and β_2M, and are mean of triplicates.

2.5.7.3 Effect of stress gene expression on hepatocyte viability and liver-specific gene expression

Hepatocyte viability was measured at the time of isolation by the UKHTB before transport to Edinburgh, and after transport and arrival in Edinburgh. Stress gene mRNA expression was determined after transport to Edinburgh, and 12-hours after plating in Edinburgh. The relationship between expression of p21, HIF1A and HSP70 and hepatocyte viability was investigated, as it was hypothesised that the two factors may be linked. For p21 and HIF1A no relationship between the two variables were found. However, a significant correlation was found between HSP-70 expression after plating and viability before and after transport (Spearman correlation r=0.6061, p-value=0.03 for the HSP-70 and viability before transport; and r=0.7418, p-value = 0.004 for HSP-70 and viability determined after transport) (see figure 2.16). The lower the cell viability the lower the HSP-70 expression suggesting that HSP-70 expression has a protective effect.

The relationship between the expression of stress genes and liver-specific genes was investigated and no correlation was found between the expression of HIF1A, p21 or HSP-70 and any of the liver-specific genes investigated.

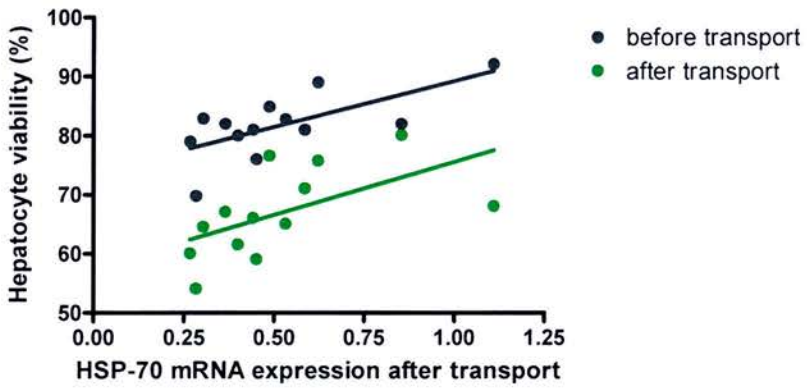


Figure 2.16: Correlation between HSP-70 mRNA expression and hepatocyte viability
The relationship between hepatocyte viability before and after transport, and normalised HSP-70 mRNA expression was investigated.

2.6 OPTIMISATION OF FRESH HEPATOCYTE TRANSPORT

The results presented in section 2.3.1 demonstrate that the viability of hepatocytes decreased between isolation and arrival at the laboratory in Edinburgh. For several research purposes fresh human hepatocytes must be transported to the place of research. All of the fresh hepatocytes used in these studies for cytotoxicity testing were sourced externally from the UKHTB (couriered to and from the airport by car and transported by aeroplane), and were transported in the UKHTB transport media (DMEM).

The initial viability significantly correlated with viability after transport (Spearman correlation: r = 0.71, p-value = 0.0065) in the hepatocytes that were subsequently used for cytotoxicity testing, after transport in DMEM (see figure 2.17). As the initial viability increases so too does the viability after transport. Because viability is a fundamental issue in research using fresh hepatocytes, and because transport of the isolated cells was a requirement for these studies, the effect of the media used for the transport and the time of transport on fresh human hepatocytes was investigated. The impact on cell viability and gene expression was investigated, as the media used for transport may influence maintenance of both viability and gene expression. The best media will provide the best environment for preservation of the cells and minimise loss of viability and maintain gene expression.

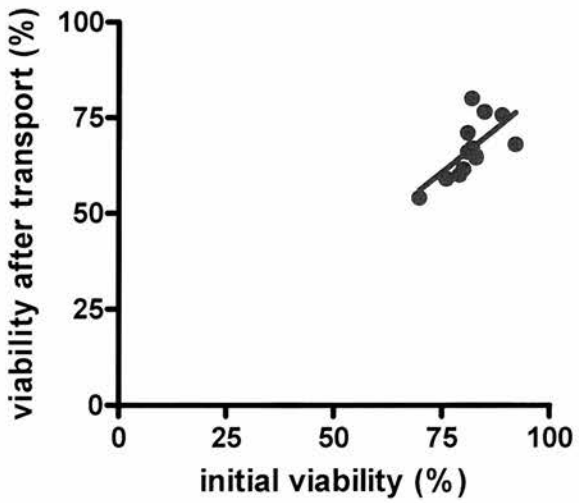


Figure 2.17: Impact of initial hepatocyte viability on viability after transport
Cell viability was determined by trypan blue exclusion immediately after isolation, and after transport in DMEM, in hepatocytes from donors 1-13.

There are several cold storage media available for the transport of isolated organs to be used for transplantation, and these media may also be used to transport isolated cells. The media investigated in these studies were DMEM, Celsior (CS), and University of Wisconsin (UW) solution. CS and UW media have been compared previously for the preservation of whole livers to be used for transplantation, and were found to impact on liver preservation but the authors concluded that a larger patient cohort was required to determine the most suitable media (Cavallari et al, 2003). Abrahamse et al (2003) showed that induction of necrosis and DNA fragmentation during hypothermic preservation of porcine hepatocytes was reduced by storage in UW and Celsior media when compared with two other storage solutions (histidine-tryptophan-ketoglutarate media or phosphate-buffered saline).

Following the observation of the loss of viability after transport in DMEM of the hepatocytes used for cytotoxicity testing, a separate set of samples were transported in 3 different media to assess the influence of the cold storage media on hepatocyte viability and gene expression. The correlation previously established between initial viability and viability after transport with DMEM medium was confirmed when pooling all samples ($r=0.44$ and $p=0.03$) (see figure 2.18). In contrast to earlier studies, when considering the subset of hepatocytes from the 11 donors tested using three different media, there was no significant correlation found between viability at isolation and viability after transport in any of the three transport media. This is likely due to the greater range of initial viabilities in the samples tested.

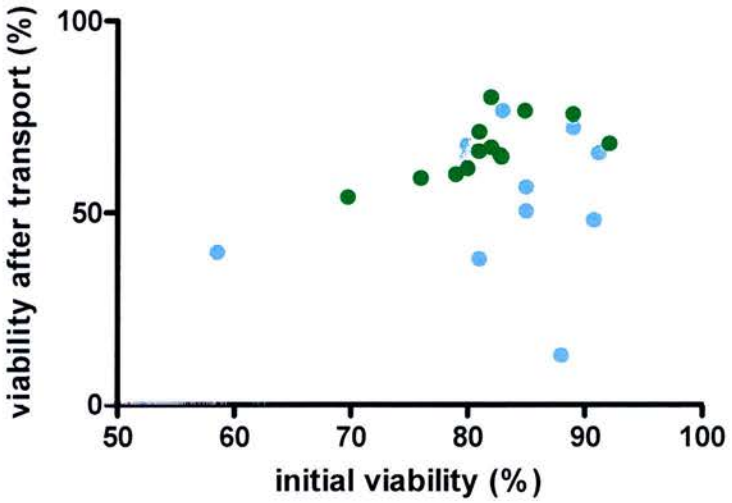


Figure 2.18: Impact of initial hepatocyte viability on viability after transport
Cell viability was determined by trypan blue exclusion immediately after isolation, and after transport in DMEM hepatocytes from donors 1-13 (green) and T1-T11 (blue).

2.6.1 Impact of transport media on hepatocyte viability

The impact of transport media on hepatocyte viability was assessed. Because the initial viability varied from donor to donor, the viability determined after transport is expressed as a percentage of the initial viability of hepatocytes isolated from that donor. UW and CS maintained the viability of hepatocytes better than DMEM during transport (figure 2.19). The loss in viability was 11-48% and 1-40% for CS and UW respectively. DMEM did not maintain viability in the hepatocytes as well as the other media, and resulted in a loss of viability of 8-85%. A non-parametric Friedman test revealed that the median of the 3 paired groups were significantly different ($p=0.01$) and with Dunn's Multiple Comparison post-test revealed that the only pair of results when compared that were significantly different ($P<0.05$) were when comparing UW and DMEM, indicating that the differences in the mean viability (expressed as % of initial viability) between these two groups is not likely due to chance. Therefore, for future studies UW would appear to be preferable for the transport of hepatocytes when compared to DMEM, due to the maintenance of hepatocyte viability.

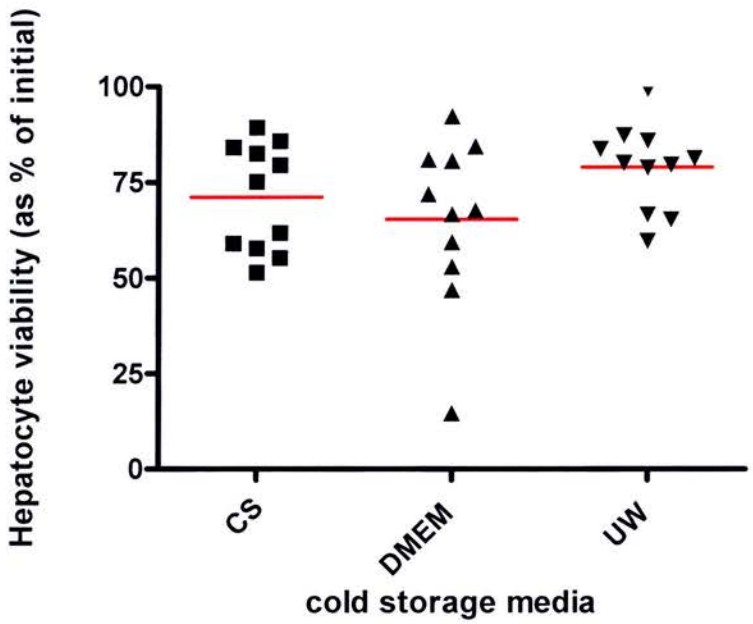


Figure 2.19: Impact of transport media on hepatocyte viability
Viability of fresh human hepatocytes (donors T1-T11) after transport on melting ice in three different transport media: CS, DMEM, or UW. Cell viability was determined by trypan blue exclusion, and is expressed as a percentage of the cell viability determined when the cells were initially isolated. Red lines indicate the mean.

2.6.2 Impact of transport time on hepatocyte viability

Because the source of the liver used for hepatocyte isolation may not be at the place of isolation and because the cells are then transported further after isolation, the time taken from liver resection to the final researcher receiving the isolated cells varies. The time of transport for cells used in these studies varied from 6.5 to 26.5 hours, and the impact of time in transport on loss of viability was investigated. Per donor the transport time does not vary. Time of transport in UW did not significantly correlate with loss of viability during transport (Spearman correlation coefficient r=0.2661, p=-0.43), however there was a significant correlation between time of transport in Celsior or DMEM and loss of viability (see figure 2.20) (Spearman correlation r= 0.8074 and 0.6698 and p=0.003 and 0.02 for Celsior and DMEM respectively). For hepatocytes transported in Celsior or DMEM, as the time in transport increased, so too did the loss of cell viability. These findings confirm that UW is the most suitable media for maintenance of hepatocyte viability during transport, irrespective of transport time.

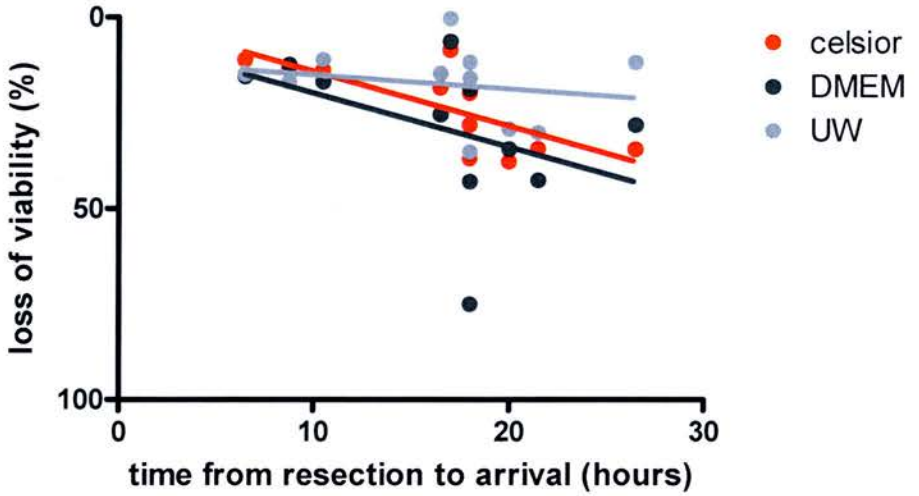


Figure 2.20: Time in transport vs. loss of hepatocyte viability
Cell viability was assessed by trypan blue exclusion immediately following isolation of hepatocytes and after transport in the different sold storage media on melting ice. The time taken from resection of the liver to receiving them is plotted against percentage loss of hepatocyte viability (initial viability minus viability after transport).

2.6.3 Impact of transport time on hepatocyte gene expression

Gene expression of AAT, ALB, CYP1A1, CYP3A4, TTR, UGT1A*, UGT2B*, and p21 was determined by qRT-PCR at the same time-points and under the same conditions as cell viability. RNA was prepared from the 'transport' set of donors (T1-T11) as described in chapter 6. For 8 out of 11 donors RNA was prepared at the time of initial hepatocyte isolation at the UKHTB, as well as after transport in Celsior, DMEM, or UW. For the other 3 out of 11 donors unfortunately this initial RNA sample is unavailable.

The impact of time in transport on gene expression of several liver-specific genes, and the stress-induced gene p21 was assessed. RNA was extracted from cells at the time of initial isolation (hepatocytes mixed with Tri® reagent by staff at the UKHTB) and from cells put into Tri® reagent after transport in Celsior, DMEM or UW to Edinburgh. The change in gene expression was calculated as a percentage of the expression after transport to the expression at initial isolation. There was no significant correlation between time in transport and gene expression for any of the genes investigated in hepatocytes transported in Celsior, DMEM or UW (see table 2.8).

Table 2.8: Spearman correlation between time from resection and change in mRNA expression (P≤0.002 considered significant following Bonferroni correction)

GENE	Celsior		DMEM		UW	
	Spearman Correlation (r)	p-value	Spearman Correlation (r)	p-value	Spearman Correlation (r)	p-value
AAT	-0.3904	0.33 (ns)	-0.2162	0.66 (ns)	-0.2440	0.58 (ns)
ALB	0.6831	0.07 (ns)	0.3416	0.39 (ns)	0.6831	0.07 (ns)
CYP1A1	-0.7563	0.04 (ns)	-0.4636	0.24 (ns)	0.6587	0.08 (ns)
CYP3A4	-0.7807	0.03 (ns)	-0.5367	0.17 (ns)	-0.0976	0.84 (ns)
TTR	-0.2196	0.62 (ns)	0.3172	0.43 (ns)	-0.2440	0.98 (ns)
UGT1A*	-0.6831	0.07 (ns)	-0.2684	0.50 (ns)	-0.2684	0.50 (ns)
UGT2B*	-0.7319	0.05 (ns)	-0.3904	0.33 (ns)	-0.5367	0.17 (ns)
p21	0.3904	0.33 (ns)	0.0976	0.84 (ns)	0.4148	0.30 (ns)

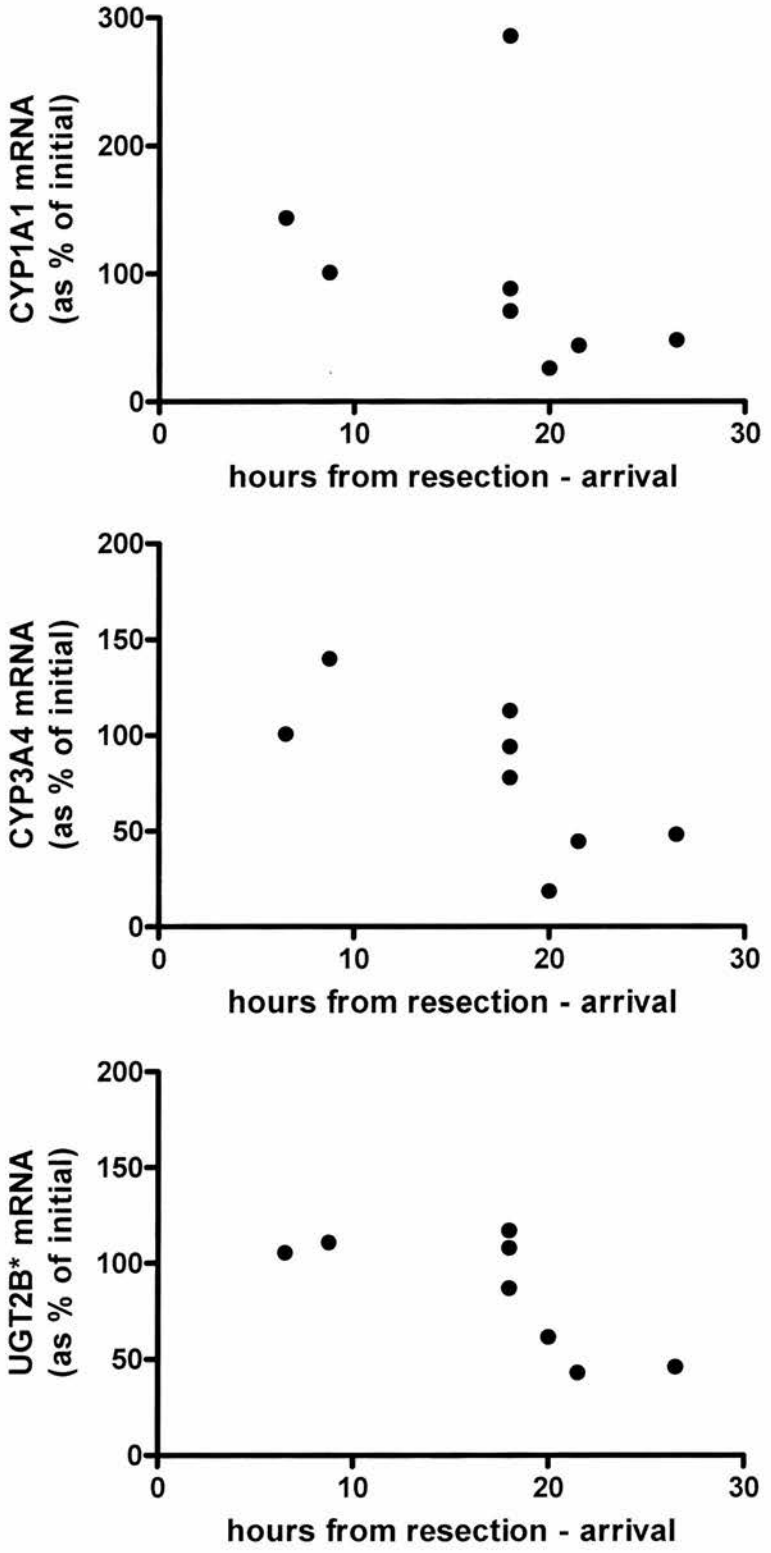


Figure 2.21: Influence of transport time on CYP1A1, CYP3A4 and UGT2B* expression

Fresh hepatocytes were transported in Celsior solution for varying lengths of time. Time in Celsior for transport negatively correlates with maintenance of mRNA expression of CYP1A1, CYP3A4 and UGT2B*.

2.6.4 Impact of transport media on hepatocyte gene expression

The impact of transport media on hepatocyte gene expression was also investigated (see figures 2.22 to 2.23). When comparing the effect of the 3 transport media on hepatocyte gene expression, no statistically significant differences were found between any of the groups using a non-parametric Friedman test. Similarly, when comparing the change in gene expression no statistically significant differences were found between any of the groups using a non-parametric Friedman test.

Normalised AAT expression was >1 (range= 1.1-2.4) after initial isolation in hepatocytes from the 8 donors investigated. AAT mRNA expression varied widely among hepatocytes stored after transport in CS from 0.4-4.1, in DMEM from 0.05 to 5.2 and in UW from 0.4-3.9. When considering the changes in gene expression for each donor, CS maintained AAT mRNA expression after transport at 37-180% of the initial value in hepatocytes, DMEM from 3-188% and UW from 34-166%. Following transport AAT expression was maintained at 50% or greater of the initial value in 6/8 donors transported in CS, 5/8 donors transported in DMEM, and 7/8 donors transported in UW. There were no significant differences detected between hepatocytes transported in either of the 3 transport media when considering AAT expression as determined by a non-parametric Friedman test. No differences were found for any of the investigated genes using the same statistical testing.

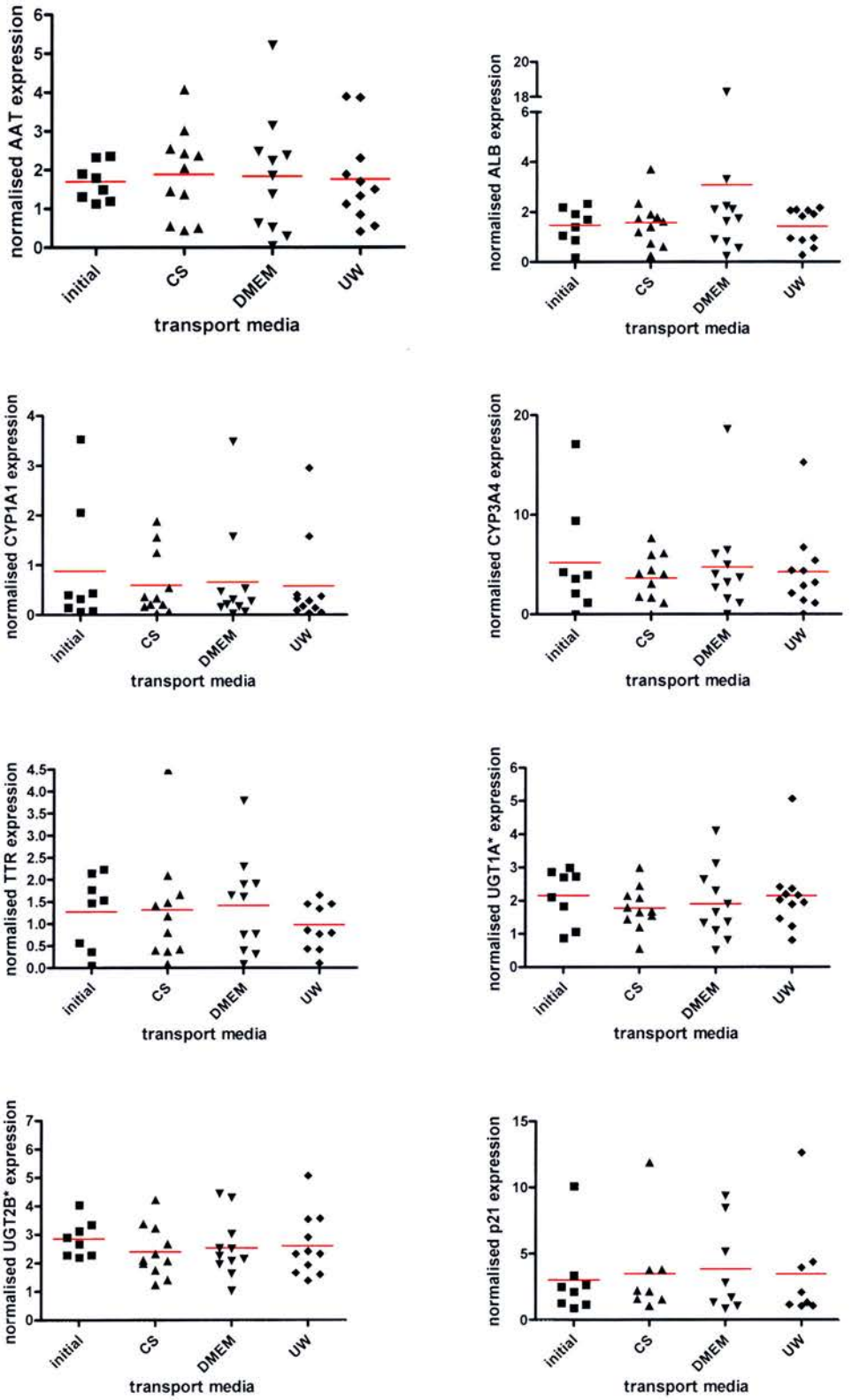


Figure 2.22: Gene expression before and after transport in Celsior, DMEM, and UW

RNA was prepared from hepatocytes initially after isolation (donors T1, 2, 5, 6, 8, 9, 10, 11) and after transport in CS, DMEM or UW (T1-11). mRNA expression is normalised to geomean of GAPDH and β2M.

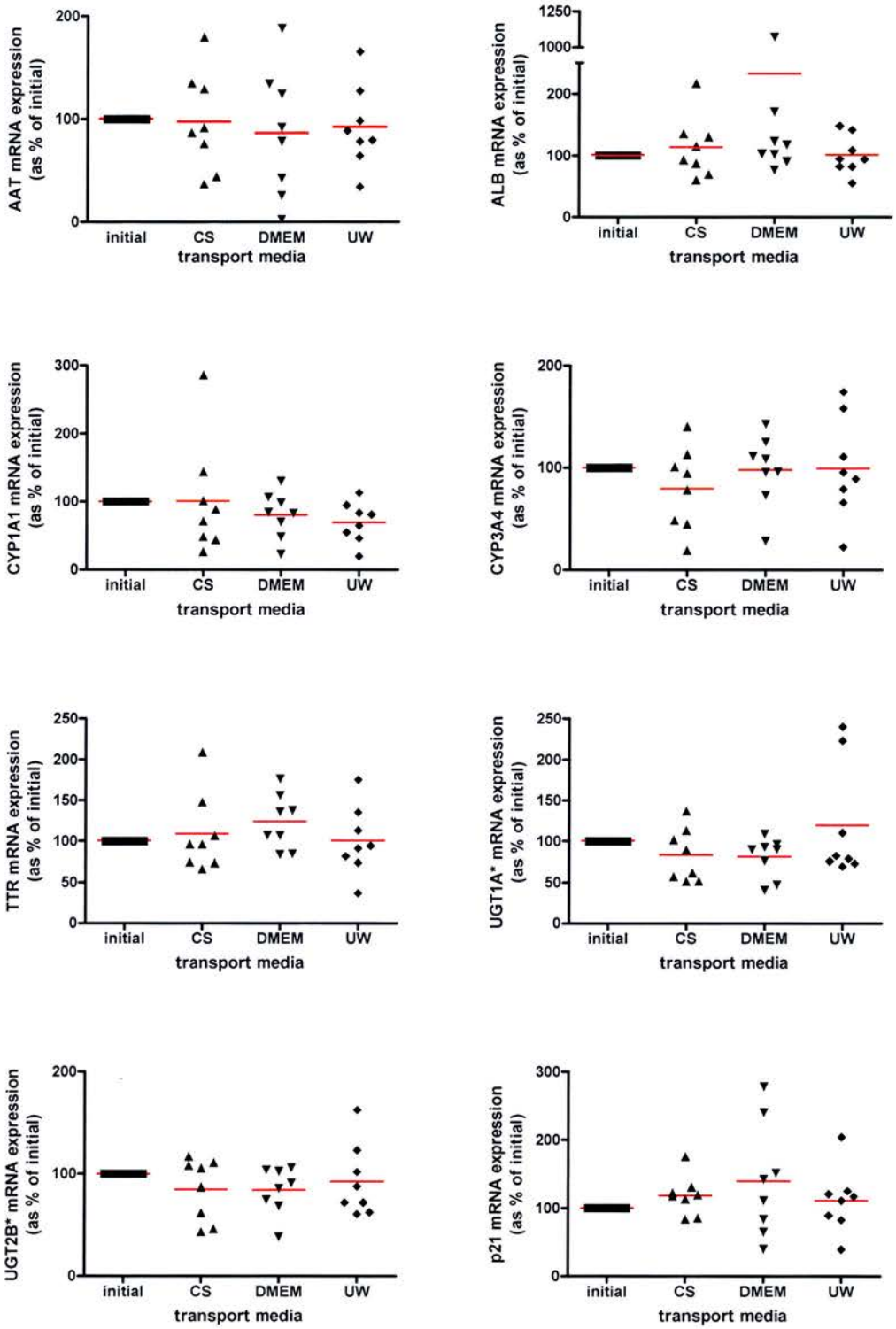


Figure 2.23: Gene expression before and after transport in Celsior, DMEM, and UW

RNA was prepared from hepatocytes initially after isolation (donors T1, 2, 5, 6, 8, 9, 10, 11) and after transport in CS, DMEM or UW (T1-11). mRNA expression expressed as a percentage of mRNA expression initially after isolation in donors T1, 2, 5, 6, 8, 9, 10, 11.

2.7 DISCUSSION

In vitro models of the liver play a vital role in many pharmacological studies, including those of metabolism and toxicity, and the study of toxicity is the purpose of the work contained in these studies. These models can be used during the development of a drug to try and detect or predict toxicity before trials of a new compound in humans, or after use in humans to investigate mechanisms of toxicity. The question of how relevant *in vitro* laboratory-based models are to the *in vivo* situation in animals and to humans is often raised and requires consideration. Different models may be more suitable depending on the investigation in question, and the determination of the most appropriate system depends on several factors ultimately decided by the needs of the user. When choosing a model and considering what may be the most appropriate to represent the liver, thought must be given to several factors. There is a need for the artificial system to accurately reflect the situation it represents. However, in some situations the most appropriate model is not just the most similar. Consideration should also be given to cost, ease of use, and reproducibility and there are also ethical considerations when using tissue from animals or humans.

One of the first considerations is whether to use an animal or human system, and there are several studies that point towards human systems being more representative of some toxicities detected in humans than animal-derived models. For example, for several of the dose-limiting toxicities caused by anticancer drugs, pre-clinical testing in rodents was found to be useful in detecting toxicities that were later revealed in patients (7/7 haematological and 3/3 neurological toxicities detected), however pre-clinical testing in *in vivo* rodent models was not predictive for the topic of this thesis, hepatotoxicity (Newell et al, 1999). Conversely, when studying the predictive value of pre-clinical toxicology studies with 12 anti-cancer platinum analogues, 6 drugs caused elevated liver enzymes in dogs and no hepatotoxicity was detected during phase I studies demonstrating hypersensitivity of the pre-clinical model (Clark et al, 1999). These examples illustrate misleading results from pre-clinical testing of anti-cancer agents in animals, and suggest a need for using human systems for investigation of *in vitro* detection of the hepatotoxic potential of anti-cancer agents.

Despite the variety of models to represent the liver and the multitude of compounds that have been tested in preclinical models, there are few published comparisons as to which models are relevant to study hepatotoxicity in humans (Guillouzo, 1998; Dambach et al, 2005). Following a thorough review of the literature, no publications were found that considered the most appropriate model for detection of hepatotoxicity caused by anti-cancer agents. Studies using one cell type *in vitro* (e.g. hepatocytes) offer the substantial advantage of being a simple model, and therefore detection of toxicity and the study of the mechanism of toxicity can be undertaken without the influence of interfering factors. However, there are several cell types in the liver, and undoubtedly one of the biggest problems in using only one cell type to detect toxicity is that any interactions between cell types that may be involved in eliciting a toxic drug response are missed. It is acknowledged that drugs can target other cells and cause toxicity to other cells in the liver resulting in other types of toxicity for example biliary tract toxicity (e.g. ecteinascidin-743) or veno-occlusive disease (e.g. actinomycin). The only human models that are available for studying more than one cell type in the liver are liver slices (Olinga et al, 1997) and co-cultures usually of hepatocytes with another cell type. However, hepatocytes are the predominant cell type in the liver and are a relevant model in which to test hepatotoxicity. *In vitro* hepatocyte-directed toxicity models are considered in these studies.

There are many published studies in which the mechanism of drug induced hepatotoxicity is investigated in only one hepatoma cell line, for example troglitazone in HepG2, paracetamol in Hep3B, and cisplatin in HepG2 (Tirmenstein et al, 2002; Manov et al, 2002; Lu and Cederbaum, 2005) without consideration of how the characteristics of the model may affect the results obtained. Davila et al (1998) reviewed *in vitro* model systems for prediction of liver toxicity. Isolated hepatocytes, whether they are fresh or cryopreserved, are a widely accepted model for many types of studies, and play a vital part in much research of the liver. Therefore, several types of model have been considered and characterised in this chapter as possible predictors of anti-cancer drug-induced hepatotoxicity, including

fresh and cryopreserved hepatocytes, hepatoma cell lines and an immortalised hepatocyte cell line.

2.7.1 Hepatocyte Viability, Attachment, and Culture

Following isolation, hepatocyte viability varied from donor to donor, and between the fresh and cryopreserved hepatocytes. The variation seen in viability of the fresh hepatocytes used in these studies is commonly encountered in studies using fresh hepatocytes (Wilkening et al, 2003; Blanchard et al, 2005, Ostrowska et al, 2000). The experimental procedure used for isolation of hepatocytes from liver tissue (two-step collagenase perfusion) involves perfusion with an isotonic buffer to remove blood and loosen cell-cell junctions (Step 1) followed by perfusion with isotonic collagenase solution to dissociate the liver parenchyma into single cells (Step 2) (Li et al, 1992). It has been reported that factors such as the quality of the liver tissue used, the composition of the isotonic buffer, and the protease composition of the collagenase used for hepatocyte isolation can effect the viability of the hepatocytes isolated (Mitry et al, 2002; Li, 2007). The perfusion buffer and collagenase solution used for hepatocyte isolation were the same for all donors in these studies; therefore the composition of these is not accountable for the variation in viability seen in these studies. Following hepatocyte isolation, the cells for these studies required transport to our laboratory, the duration of which lasted 6.5-26.5 hours. Hepatocyte viability was found to drop significantly during transport on ice in DMEM. The cryo-preserved hepatocytes from 4 donors included in these studies had significantly lower viability after recovery from cryopreservation than the same cells before cryopreservation and the fresh hepatocytes (before and after transport). The loss of hepatocyte viability following cryopreservation has been widely acknowledged (Hengstler et al, 2000; Terry et al, 2005), and research is ongoing for techniques to improve cryopreservation and utility of hepatocytes to increase the use of this valuable research resource.

The source of hepatocytes (from 12/13 donors) for the studies presented here was the healthy tissue resected at the time of surgery for liver metastases. The effect of donor characteristics on hepatocyte viability was assessed, and no correlation was

found between sex, age, or smoking status of the donors and the viability of isolated hepatocytes used in these studies. The retrospective analysis of donor characteristics in 149 patients on the outcome of isolated hepatocytes by Alexandre et al (2002), also revealed no effect of age and sex of the patient or tobacco consumption on viability of isolated human hepatocytes. The study by Alexandre et al also demonstrated that additional factors (e.g. previous chemotherapy and alcohol consumption) did not affect yield, viability, attachment rate and function of the isolated hepatocytes. A further study by Hewes et al (2006) confirmed that pre-resection chemotherapy does not affect the integrity of hepatocytes isolated from healthy liver tissue after resection for colorectal liver metastases (in 47 patients studied), which is of key importance considering the source of the fresh hepatocytes used in these investigations.

Mitry et al (2002) report that under standard *in vitro* cell culture conditions, mature hepatocytes usually survive for 10-14 days. Whilst the cells can survive for this length of time in culture, maintenance of liver specific functions can only be maintained for approximately one week (Gomez-Lechon et al, 2003) and the culture conditions required for this include the presence of an extracellular matrix and media supplementation. These factors were incorporated in this experimental design, with hepatocytes (fresh and cryopreserved) plated on collagen-I pre-coated flasks and media supplemented with dexamethasone and insulin (LeCluyse et al, 2005). The maximum length of experiment, and therefore maximum time for hepatocytes in culture was 108 hours.

For the purpose of the studies presented here, the ability of cells to adhere to their tissue culture substratum is imperative. This is one of the fundamental criteria for the assays used for detection of response of these cells to anti-cancer agents (that are presented in chapters 3 and 4). Additionally, hepatocytes in suspension can only survive for short periods of time in culture. Not all hepatocytes maintain their adherence capabilities after cryopreservation, and only the cryopreserved hepatocyte batches that attached to the pre-coated tissue culture plates were suitable for use in these assays. Terry et al (2005) found that the source from which the hepatocytes

were isolated can impact on parameters including viability after thawing, attachment efficiency and albumin production which could explain why some of the cryopreserved hepatocytes used in these studies never attached.

2.7.2 Cell Proliferation

The liver is normally a quiescent organ, which makes the establishment of a reliable and reproducible model in which to study hepatic toxicity problematic. Arguably the most pronounced difference between fresh or cryopreserved hepatocytes and the cell lines investigated is that the hepatocytes do not proliferate *in vitro* under standard culture conditions as used for these studies (Gomez-Lechon et al, 2003), whereas the other models investigated do. All of the hepatoma cell lines replicated in culture and the doubling times varied. The THLE-2 cell line was found to be able to replicate in culture as reported by Pfeifer et al (1993), and had a doubling time similar to Hep3B2.1-7: the longest doubling time of the hepatoma cell lines. Proliferating cells are more appealing to work with *in vitro* in that a supply is readily available and costs are greatly reduced compared with primary human hepatocytes. However they are not quiescent like the liver. The cellular targets of anti-cancer drugs vary, however the primary mode of action is to target proliferating cells and consequentially cause cell death. Therefore the choice between a proliferating and non-proliferating cell line becomes even more critical when detecting liver toxicity with anti-cancer drugs. The cytotoxicity of anticancer agents targeting DNA may be overestimated in proliferating cell lines as compared to fresh hepatocytes, because their target is DNA and DNA replication will be ongoing in cell lines. This may have implications when considering whether or not to select a replicating or non-replicating cell model.

2.7.3 Liver gene expression

Detection of mRNA levels by qRT-PCR was used as a technique to compare the models in these studies. The cost of microarray and proteomic technologies and the quantity of RNA and protein required for these techniques were prohibitive for this work. The gene expression analysis has revealed several differences in liver-specific genes between the *in vitro* models. In summary, the fresh hepatocytes expressed all

of the liver specific genes investigated at detectable levels, albeit with variation between donors and a time-dependent decrease of mRNA content in hepatocytes from the majority of donors for the majority of genes. The cryopreserved hepatocytes also expressed all liver-specific genes investigated at detectable levels, with the level of expression of 4 genes involved in drug metabolism and transport (ABCB1, CYP3A4, UGT1A* and UGT2B*) being comparable with the fresh hepatocytes from some donors. Two of the hepatoma cell lines (PLC/PRF/5 and SK-HEP-1) and THLE-2 cells had no detectable expression of the liver-specific genes with the exception of AAT, AFP and CYP1A1 expression in PLC/PRF/5, and AAT and CYP1A1 expression in THLE-2 cells. The remaining three hepatoma cell lines (Hep3B2.1-7, Huh-7D12 and Hep G2) expressed 7 or more out of the 10 liver-specific genes investigated. AFP was only found expressed in the hepatoma cell lines, with the highest expression being in Hep G2 and Huh-7D12 and lower expression detected in Hep3B2.1-7 and PLC/PRF/5. Therefore based on the gene expression data obtained, fresh hepatocytes would be the best model to represent the liver, followed by the cryopreserved hepatocytes. Of the replicating cell lines, Hep3B2.1-7 would appear the most appropriate as it expressed 7/10 of the genes investigated and had lower levels of AFP than Hep G2 and Huh-7D12.

With regards to the gene expression studies, the standards used for relative quantification were the same for all cell types for each gene. RT-PCR gene expression is expressed relative to commercially available RNA from one 51-year old male Caucasian liver for all genes except AFP where foetal liver (pooled from 63 spontaneously aborted male/female Caucasian foetuses, ages 22-40 weeks) is used. For some genes it is possible that the reference standard may have particularly high or low expression of a particular gene. For example normalised ABCB1 expression in hepatocytes from all 12 donors detected was >1 so it is possible that the standard had low ABCB1 expression. The reference RNA is made from whole liver as opposed to hepatocytes and could also explain higher relative expression in hepatocyte samples compared to the standard for many of the genes investigated. It is acknowledged that the gene expression considered to be closest to that of human

liver is that in isolated hepatocytes. It is unknown if the procedure of hepatocyte isolation alters the gene expression to that *in situ*.

The mRNA expression data obtained demonstrated variability in all genes in fresh hepatocytes from 13 donors. For some genes there were outliers whose expression was very different from other donors, for example CYP1A1 expression in donor 12. CYP1A1 is an inducible CYP isoform by inducers such as polyaromatic hydrocarbons, however donor 12 was not a known smoker and it not known that donor 12 was exposed to any other CYP1A1 inducers. Dexamethasone is used as an additive in the culture media and is a CYP3A4-inducer (Roymans et al, 2005) and could therefore explain the relative higher expression of CYP3A4 in 8 out of 12 donors when compared with the reference standard RNA, despite the drop in CYP3A4 in all donors from arrival time to 12-hours after plating. Loss of CYP3A4 gene expression during culture follows the same trend as protein and activity levels decline, however the decrease in mRNA levels precedes CYP protein content and activity loss (Gomez-Lechon et al, 2003). Additionally, because CYP regulation is predominantly pre-translational, mRNA levels allow a good estimation of CYP activity (Gomez-Lechon et al, 2003).

It may also be possible to assess the requirement of CYP activation of a drug to toxic metabolite(s) for toxicity induction by the use of CYP-nonexpressing hepatoma cell lines (ie ready-made knockout).

Pfeifer et al (1993) generated the THLE-2 cell line from human liver epithelial cells and reported expression of hepatocyte phenotypic traits. These included expression of cytokeratins 18 and 19, AAT and albumin secretion, and no AFP expression at early passage. By qRT-PCR, the only assessed genes that were detectable in THLE-2 cells were AAT and CYP1A1, but they were expressed at lower levels than in the hepatocytes (50-fold and 33-fold lower than in the reference RNA, respectively). There are very few other published papers with data obtained using the THLE-2 cell line. The cell lines SK-HEP-1 and THLE-2 that express 0 and 2 genes respectively may therefore be considered less suitable models to represent the liver *in vitro*.

In summary, there are fundamental differences in expression between the models investigated, which may influence the potential of these models to detect drug-induced toxicity. For example with drugs that are metabolised by CYP3A4, use of a model with no CYP3A4 expression (e.g. THLE-2, HepG2, Huh-7D12, PLC/PRF/5 or SK-HEP-1) may result in overestimation of cytotoxicity due to lack of detoxifying drug metabolism. Conversely, if a drug were metabolised by CYP3A4 to a toxic metabolite, choice of a model lacking expression would under-predict toxicity due to lack of activation of drug. However, metabolism could be evaluated prior to toxicity testing using microsomal systems. Similarly, high ABCB1 expression (e.g. fresh hepatocytes) may lead to under-prediction of toxicity if drug is transported out of cells at a faster rate than would occur in humans, and an ABCB1 non-expressing model (THLE-2, PLC/PRF/5 or SK-HEP-1) may over-predict hepatotoxicity due to build up of drug within hepatocytes. These examples demonstrate how knowledge of gene expression may aid interpretation of data generated using these *in vitro* models.

2.7.4 Stress gene expression

The analysis of p21, HSP-70 and HIF1α mRNA revealed an increase in p21, HSP-70 and HIF1α expression following plating in hepatocytes from all donors except donor 12 for which p21 mRNA expression decreased. One factor that could influence the increase in stress gene expression is the change in temperature following the isolation of hepatocytes. The liver will be removed from the body at 37°C, stored at 4°C until hepatocyte isolation, warmed to 37°C during isolation, transported on melting ice and then transferred to an incubator once plated in media at 37°C. The removal of the liver from the body requires stoppage of blood flow to the isolated part of the liver being removed, and means the liver will no-longer be exposed to circulating factors and nutrients in the blood. This, along with disruption of the normal liver architecture, and an adaptation to *in vitro* culture conditions could result in increased stress-induced gene expression.

An interesting correlation was found between HSP70 expression and hepatocyte viability. The higher the cell viability both before and after transport, the higher the

HSP70 expression, suggesting that HSP-70 expression may protect against cell death. Increased expression of HSPs has been shown to be protective in many cultured cells and animal tissues under a variety of conditions (Kiang and Tsokos, 1998, Hassen et al, 2005). HSP-70 is inducible and induction following plating may be due to an increase in transcription after transport. The correlation was found using hepatocytes from 13 donors which is a small sample size. If this correlation can be confirmed in a larger cohort of patients it may be useful to consider the development of HSP-70 agonists to maintain hepatocyte viability either for use in *in vitro* assays or it may help in the development of cells for transplant in humans. There are several similarities between HSP-70 and HSP-90 and there are HSP-90 inhibitors currently in clinical development for the treatment of cancer (Vastag, 2006). For example, 17-allylamino, 17-demethoxygeldanamycin was the first HSP-90 inhibitor to enter clinical trials because the first pre-clinically studied HSP-90 inhibitor geldanamycin was not tested in humans due to the detection of hepatotoxicity pre-clinically.

HSP-70 expression is linked to cell viability in the fresh hepatocytes. Intriguingly HSP-70 expression is higher in the cryopreserved hepatocytes than in fresh hepatocytes, and the cryopreserved hepatocytes have a lower viability. This is contradictory to the pattern observed in fresh hepatocytes, but it is likely that the cryopreservation process is responsible for the lower viability in cryopreserved hepatocytes. It is possible that only the cryopreserved hepatocytes with higher HSP-70 expression survive the cryopreservation process, which is consistent with the suggestion that HSP-70 expression may protect against cell death.

2.7.5 Comparison of fresh hepatocyte transport media

Laboratories in several cities in the UK source fresh hepatocytes from the UKHTB and therefore transport is a requirement for these cells. Transport of isolated hepatocytes is required throughout the world where laboratories do not have the facilities and/or expertise for hepatocytes isolation. The choice of media used for storage and transport of organs for transplantation influences the organ (e.g. Feng et al, 2007) and therefore it is likely that the media used for transport of isolated

hepatocytes would impact on the quality of the transported cells. Indeed, the studies reported here demonstrated a loss of viability following transport in DMEM. Therefore, the effect of 3 transport media on hepatocyte viability and gene expression was investigated. An increase or decrease in gene expression during transport is undesirable. The study of transport media revealed that UW maintained the highest viability during transport when compared with DMEM and celsior, but there were no significant differences between the 3 transport media in terms of maintenance of gene expression of any of the genes investigated. Time in transport was also considered and was found to be associated with loss of viability when hepatocytes were transported in DMEM or celsior, but to have no effect on gene expression.

Studies have previously been carried out assessing the influence of cold storage media for liver storage on the fresh hepatocytes subsequently isolated. A study by Donato et al. (2005) investigated cytochrome P450 expression in human hepatocytes isolated from human liver grafts preserved in CS prior to isolation and reported that CYP1A1 showed greater variability in mRNA expression between donors than any of the other cytochrome P450s they investigated, which did include CYP 3A4 (Donato et al, 2005).

Gene expression will impact on protein expression and therefore activity, and from these studies no difference was found between the 3 media investigated in their ability to maintain mRNA of several genes during transport.

2.7.6 Conclusions

In conclusion, the studies presented in this chapter provide a valuable comparison between the selected *in vitro* models. The similarities and dissimilarities of the different cell types have been considered, and revealed that the models differ in all parameters considered (morphology, viability, proliferation, and gene expression). Based on the findings presented in this chapter fresh hepatocytes would be considered the most similar model to hepatocytes in humans, in that they do not proliferate and they express the liver-specific genes investigated. The viability

varied between fresh hepatocyte donors and it would be desirable to maintain it at a higher level during transport, perhaps by transporting cells in UW media. The cryopreserved hepatocytes were similar to hepatocytes in humans, with no proliferation in culture and expression of liver specific genes albeit at lower levels, however methods of cryopreservation need to be developed to increase their post-thaw viability and attachment properties. Morphology, gene expression, and lack of proliferation in culture were similar in the fresh and cryopreserved human hepatocytes. These data allow more informed interpretation of further studies of drug-induced toxicity in these models.

As discussed previously, consideration of hepatotoxicity with anti-cancer agents is different from that of other classes of agents. Due to the mechanism of action of anti-cancer drugs and the fact that the liver under normal circumstances is a quiescent organ the differences in growth capabilities of these cell models may influence toxicity detection. An understanding of the differences in morphology and gene expression may aid understanding of differences in response to anti-cancer agents. With the knowledge of the basal differences between the models in the parameters considered in this chapter, anti-cancer drug treatment of the models was carried out to determine how the models respond to different anti-cancer agents.

CHAPTER 3: Results
Assessment of liver toxicity: Cytotoxicity of selected anti-cancer drugs *in vitro*

3.1 INTRODUCTION
- 3.1.1 *In vitro* Cytotoxicity Assays
- 3.1.2 Pharmacological Properties and Hepatotoxic Potential of Selected Drugs
- 3.1.3 Principle of Therapeutic Index

3.2 *IN VITRO* CYTOTOXICITY ASSAYS
- 3.2.1 Determination of Optimal Time-point to Detect Cytotoxicity
- 3.2.2 Comparison of SRB and MTT Assays

3.3 DRUG TREATMENT OF CELLS
- 3.3.1 Cytotoxicity in Colon Cancer Cell Lines
- 3.3.2 Cytotoxicity in *in vitro* Liver Models
- 3.3.3 Therapeutic Index

3.4 POTENTIAL INFLUENCES ON CYTOTOXICITY IN FRESH HEPATOCYTES
- 3.4.1 Donor Demographics
- 3.4.2 Gene Expression

3.5 KINETICS OF CELL DEATH

3.6 APPLICATION OF *IN VITRO* HEPATOTOXICITY TESTING TO DRUGS IN DEVELOPMENT
- 3.6.1 Ruthenium Compounds HC11 and RM175

3.7 DISCUSSION
- 3.7.1 *In vitro* Cytotoxicity Assays
- 3.7.2 Drugs Tested and Therapeutic Index
- 3.7.3 Influences on Cytotoxicity in Fresh Hepatocytes
- 3.7.4 Time-points Selected and Kinetics of Cell Death
- 3.7.5 Conclusions

3.1 INTRODUCTION

Using the *in vitro* models that have been considered in the previous chapter, the hepatotoxicity caused by treatment with anti-cancer drugs will be investigated. The benefits of *in vitro* tests to detect drug-induced liver toxicity relates to their minimal cost and their relatively good predictability when compared to animal testing (Xu et al, 2004). The mechanisms of cell death induced by chemicals in hepatocytes are diverse, and cell death can be considered as a suitable endpoint for studying anticancer drug-induced toxicity.

Initially three drugs were selected to assess the predictability of the *in vitro* models with the chosen assays. A known hepatotoxic drug, mithramycin, and 2 drugs used for the treatment of colorectal cancers (5-FU and oxaliplatin) were selected for treatment of the *in vitro* models.

Two *in vitro* assays were used for detection of cell death (the sulforhodamine B (SRB) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays) to measure the response of the different cells to drug treatment and to determine the best model for accurate prediction of hepatotoxicity. The most appropriate and accurate time-point for determining cytotoxicity and defining the IC_{50} was investigated. Unlike other therapeutics, anticancer agents are inherently cytotoxic. Therefore, the safety of anticancer agents is not assessed by their lack of activity against normal cells but the difference between the cytotoxic effect induced in tumour cells and normal cells (therapeutic index). As the kinetics of cell death may differ between drugs and *in vitro* models, the kinetics of cell death was also investigated for each of the three compounds in fresh hepatocytes.

The use of the *in vitro* models was then extended to include drugs in development. Novel ruthenium compounds, which have been investigated pre-clinically, were evaluated using the same assays to assess whether the *in vitro* assays would have predicted the pre-clinical toxicology data obtained in mice.

3.1.1 *In vitro* Cytotoxicity Assays

In order to study the effect of the anti-cancer drugs on cells *in vitro*, it is necessary to detect living cells. Several methods can be employed for this purpose. Cytotoxicity can be determined via assays that detect cell proliferation or viability, and are available to detect different characteristics of cells. Plasma membrane leakage may be detected using a lactate dehydrogenase release assay; metabolic activity of mitochondria may be measured using a colorimetric substrate (WST-1, XTT, and MTT) that requires conversion before detection; critical cellular macromolecules such as ATP may be detected; or total cellular protein may be determined using sulforhodamine B (SRB).

The main factors in selecting assays for use in these studies are suitability for 96-well plate format, speed, ease of use, and cost. Therefore, the SRB and MTT assays were selected because they meet these criteria, utilising colorimetric detection and being suitable for microplate format.

3.1.1.1 SRB Assay

The SRB assay provides an indirect measure of cell number by binding to and staining cellular protein in living cells (Skehan et al, 1990), and has been optimised previously (Papazisis et al, 1997). The SRB assay is routinely used by the National Cancer Institute (NCI) in the United States to screen anti-cancer drugs in their panel of 60 cancer cell lines (NCI60). This assay is described in further detail (Chapter 6, Materials and Methods) and its linearity was confirmed by plating a range of cell densities as a calibration curve. Due to technical difficulties using the SRB assay with fresh hepatocytes, the MTT assay was also investigated and used with the fresh hepatocytes.

3.1.1.2 MTT Assay

The MTT assay provides direct measurement of cell viability. MTT is a yellow water-soluble tetrazolium dye that is reduced by the mitochondria of viable living cells to a water-insoluble purple formazan (Banasiak et al, 1999). The amount of formazan can be determined by solubilising the purple-formazan in DMSO and

measuring the OD spectrophotometrically. The ratio of MTT OD in drug-treated cells compared to control cells allows determination of the surviving fraction of cells at a particular drug concentration. By plotting the fraction of surviving cells against drug concentration, determination of the drug concentration killing 50% of the cells (IC_{50}) is possible (Alley et al, 1988). This assay will be described in Chapter 6, Materials and Methods and its linearity was confirmed by plating a range of cell densities as a calibration curve.

3.1.2 Pharmacological Properties and Hepatotoxic Potential of Selected Drugs

Mithramycin, oxaliplatin and 5-FU were chosen as anticancer drugs to test the *in vitro* models for the prediction of hepatotoxicity due to the reported presence or absence of liver toxicity in patients following treatment in the clinic with these drugs.

3.1.2.1 5-fluorouracil

5-FU is a fluorinated analogue of uracil used for the treatment of common solid tumours, and rapidly enters the cell using the same facilitated transport mechanism as uracil. The chemical structure of 5-FU is shown in figure 3.1.

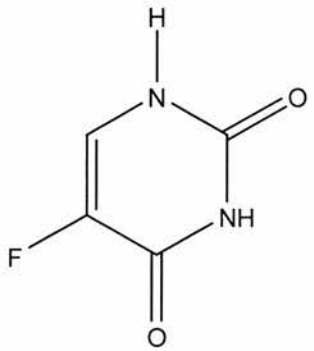


Figure 3.1: Chemical structure of 5-FU

5-FU follows the same metabolic pathway as uracil, forming fluorinated deoxyribonucleotides incorporated into DNA, fluorinated nucleotides incorporated into RNA and FdUMP, and is an inhibitor of thymidylate synthase (TS). 5-FU is converted intra-cellularly to several active metabolites, which disrupt RNA synthesis and the action of TS. Incorporation of 5-FU metabolites into RNA and DNA and

inhibition of TS eventually leads to transcriptional error and arrest, ultimately resulting in cell death and hence the drug's anticancer action.

5-FU is extensively inactivated by the enzyme dihydropyrimidine dehydrogenase (DPD) into dihydrofluorouracil. DPD is abundantly expressed in the liver, and over 80% of administered 5-FU is catabolised in the liver (Longley et al, 2003). Patients genetically deficient in DPD can experience severe toxicities with 5-FU (Raida et al, 2001). Because of the role of the liver in 5-FU catabolism, the liver is a relevant target organ for drug toxicity. 5-FU has been reported to cause mild and infrequent liver toxicity in patients identified by increases in amino-transferases (Witte et al, 2001) or the development of fatty infiltration of the liver (steatosis) (Peppercorn et al, 1998).

3.1.2.2 Oxaliplatin

Oxaliplatin is a platinum derivative that is mainly used in the treatment of cancer of the large bowel. Furthermore, *in vitro* oxaliplatin demonstrates activity in colorectal cancer cells (Arango et al, 2004) and in human gastric cancer cell lines (Eriguchi et al (2003). Oxaliplatin acts by creating intra- and inter-strand DNA adducts (Graham et al, 2004), and subsequently irreversible DNA replication error and cell death. The chemical structure of oxaliplatin is shown in figure 3.2. The liver does not play a major role in the metabolism of oxaliplatin and therefore would not be at any increased risk of toxicity due to its high drug metabolising capability. One study has reported that oxaliplatin is well tolerated in patients with liver dysfunction, and there was no apparent alteration in the clearance of either total or ultra-filterable platinum species from plasma, even in patients with severe hepatic functional abnormalities (Doroshow et al, 2003).

Oxaliplatin was selected as a negative control for this project; however, more recently there have been reports of oxaliplatin treatment in patients resulting in adverse effect on the liver (Tisman et al, 2004; Rubbia-Brandt et al, 2004). Pre-operative administration of oxaliplatin in patients with hepatic colorectal metastases has been noted to result in fatty change and severe steatohepatitis, especially in the

obese (Fernandez et al, 2005). Additionally, toxicity of oxaliplatin-containing neoadjuvant chemotherapy has been reported by Arotcarena et al (2006), and was detected as changes in LFTs manifesting as an increase in GGT and ALP, and when examined histologically hepatic sinusoidal lesions were present and thought to be caused by injury initially to the sinusoidal endothelial cells causing sinusoidal wall disruption (Rubbia-Brandt et al, 2004).

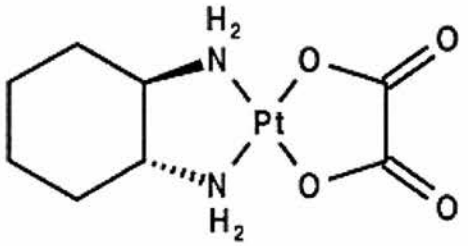


Figure 3.2: Chemical structure of oxaliplatin

3.1.2.3 Mithramycin

Mithramycin is no longer used routinely for the treatment of cancer; however it has been used for the treatment of testicular cancer in the past and can be used to treat tumour hypercalcaemia refractory to other chemotherapy (Zojer et al, 1999). The possibility of using mithramycin in combination regimens with another anticancer agent (bevacizumab) is currently being investigated (Jia et al, 2007) and therefore study of the hepatotoxicity caused by mithramycin is of interest. Mithramycin is the most hepatotoxic chemotherapeutic agent available (King and Perry, 2001), and was selected as a positive control for these studies. The chemical structure of mithramycin is shown in figure 3.3. Mithramycin is a potent inhibitor of transcription, binding to GC rich regions in promoter sequences of DNA (Miller et al, 1987), and is a potent inhibitor of RNA transcription, decreasing protein synthesis. Mithramycin can therefore block the production of many intracellular enzyme systems necessary for normal hepatic function (King and Perry, 2001). Elevations of aminotransferases and lactate dehydrogenase occur in almost all patients treated with mithramycin, and milder elevations in alkaline phosphatase levels also occur (King and Perry, 2001). These changes begin on the day of drug

administration, peak on the second day, and return to normal by 4 to 21 days after treatment.

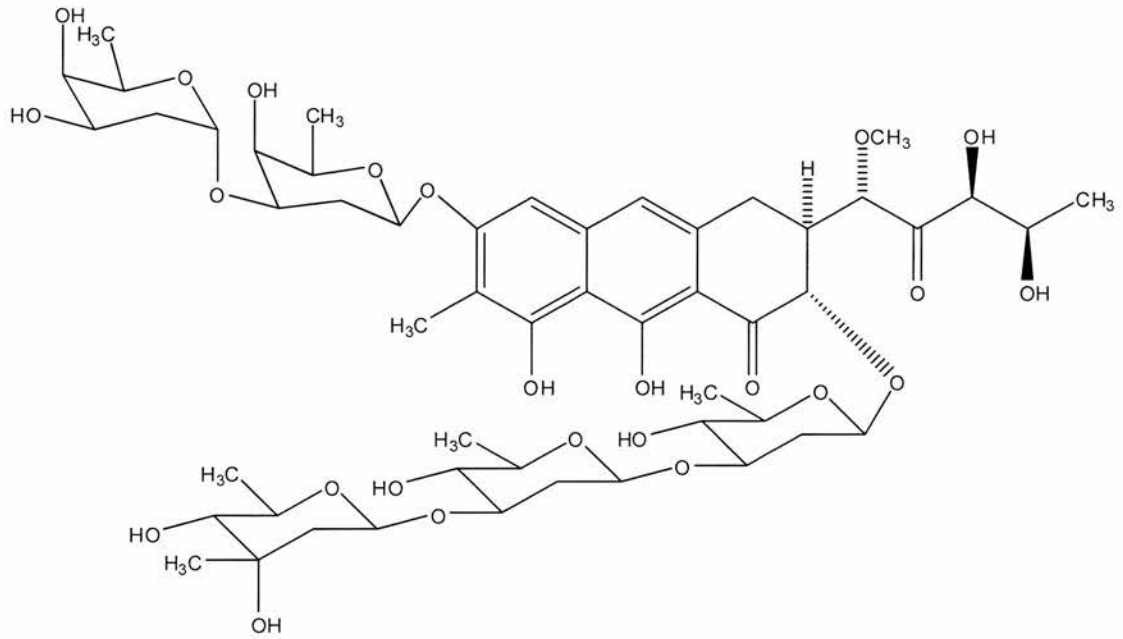
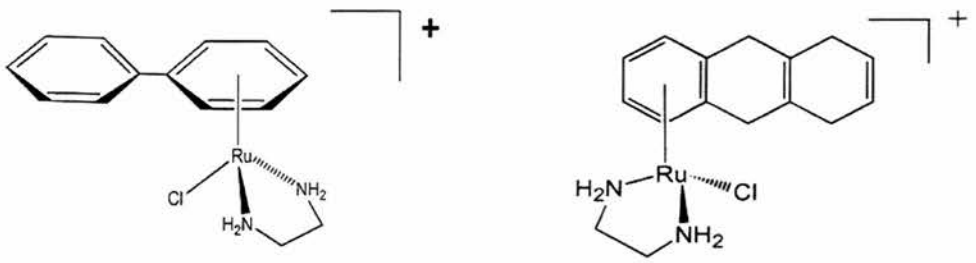


Figure 3.3: Chemical structure of mithramycin

3.1.2.4 Ruthenium compounds HC11 and RM175

Several metal-containing complexes have anti-cancer properties and those licensed for use in the UK include cisplatin, carboplatin and oxaliplatin. The metal ruthenium has recently formed the basis for development of new anti-cancer compounds (reviewed by Kostova, 2006). $[(\eta 6\text{-}C_6H_5C_6H_5)Ru(en)Cl]^+$ (RM175), and [(η6-tetrahydroanthracene)Ru(en)Cl]$^+$ (HC11), are two ruthenium-containing anti-cancer compounds investigated in this study. The chemical structures of RM175 and HC11 are shown in figure 3.4. Pre-clinical trials involving RM175 and HC11 have demonstrated activity of these compounds in ovarian cancer cell lines and small cell lung cancer cell lines (Aird et al, 2002). Brabec and Novakova (2006) investigated the DNA binding mode of ruthenium complexes and their relationship to tumour cell toxicity. Pre-clinically, liver toxicity caused by ruthenium compounds RM175 and HC11 has been detected in mice (Guichard et al, 2006). Analysis of blood from the mice revealed increased ALT, particularly following HC11 treatment, and histopathological examinations revealed necrotic foci in some of the drug-treated groups when compared to controls, following sacrifice of the animals.



RM175 **HC11**

Figure 3.4: Chemical Structure of RM175 and HC11 (Guichard et al, 2006)

3.1.3 Principle of Therapeutic Index

When determining the potential usefulness of any drug the benefits and risks must be considered. Most anti-cancer drugs cause toxicity if the administered dose is high enough. Drugs interact with a specific therapeutic target and the specificity of the reaction is based on the affinity of the drug for its target. At higher drug concentrations, interactions with targets for which the drug has lower affinity may occur, leading to 'off-target effects' which are often related to adverse drug reactions (Guengerich and MacDonald, 2006). One criterion of drugs selected for development is that they have high affinity and specificity for the drug target so as to minimise potential off-target effects.

For a drug to be useful and feasible for administration in humans, the efficacious dose must be lower than the dose that causes toxicity. The therapeutic index is defined as the difference between dose associated with toxicity and efficacious dose. During treatment with anticancer drugs it is necessary to produce maximal benefit and minimal toxicity. Anticancer drugs are designed to kill cells i.e. they are cytotoxic. For interpretation of the data produced from cytotoxicity assays presented in these studies, the principal of the therapeutic index will be considered.

Ehrlich first recognised that a drug must not be solely judged by its useful properties, but also by its toxic effects, and he described the therapeutic index (TI) of a drug in

terms of the ratio between the average minimum effective dose and the average maximum tolerated dose in a group of patients (Rang et al, 2001):

$$TI = \frac{\text{maximum tolerated dose}}{\text{minimum effective dose}}$$

However, in the definition of the TI given above, interindividual variability is not considered. A widely used definition that does take into account interindividual variability and may be defined from preclinical studies is:

$$TI = \frac{LD_{50}}{ED_{50}}$$

When defined as LD_{50} (lethal dose for 50% of a group of animals) over ED_{50} (effective dose for 50% of a group of animals), the TI gives some indication of the safety margin in the use of a drug. Of interest, the LD_{10} in pre-clinical animal studies with anti-cancer agents is considered to be the maximum tolerated dose, and one-tenth of the LD_{10} in animal studies is often used as the starting dose in humans.

For interpretation of data presented in this chapter, the principle of determining a TI from a group of animals has been adapted for description of the proportion of cells killed by drug treatment *in vitro*. To indicate the window between effective and toxic dose in these cell models, TI is defined as:

$$TI = \frac{IC_{50} \text{ in hepatocyte model (toxic)}}{IC_{50} \text{ in colon cancer model (therapeutic)};}$$

where a ratio of 1 would indicate no difference in IC_{50} between the two cell types, a ratio of <1 indicating that the drug was more toxic to the liver than in the target cell, and a ratio of >1 indicating that the IC_{50} in the hepatocyte model is higher than in the target cell and active drug concentrations may be achieved without resulting in toxicity.

3.2 *IN VITRO* ASSAYS

Two cytotoxicity assays (SRB and MTT) were used to detect cell viability *in vitro* with a variety of cell types, and the proportion of cells that were killed by drug

treatment was assessed. Assessment of the time-point at which to detect maximum cytotoxicity, and a comparison between the MTT and SRB assays was carried out.

3.2.1 Determination of Optimal Time-point to Detect Cytotoxicity

In order to determine the most appropriate timepoint for accurate detection of cell death, a time-course with measurements made every 24 hours was undertaken (starting 24 hours after addition of drug, and at 48-, 72- and 96-hours after drug addition) and measurement of cellular response to drug using SRB or MTT assays. Both the PLC/PRF/5 cell line and fresh human hepatocytes were investigated. The surviving fractions compared to controls were determined using the MTT and SRB assay every 24 hours for fresh hepatocytes and PLC/PRF/5 respectively. It was desired to define the time for IC_{50} measurement at which maximal cytotoxicity was observed.

Cells were plated and allowed to attach to the tissue culture plastic (time for attachment was dependent upon cell type) before the addition of a concentration range for each drug for 24 hours. After 24 hours, the drug was removed, cells were washed, and the MTT or SRB cytotoxicity assays performed, or media replaced and cytotoxicity assays performed every 24 hours until 96 hours after the start of drug treatment.

The cytotoxic effect increased over time using both assays in fresh hepatocytes as well as in the hepatoma cells. Figure 3.5 shows examples of dose response curves for each drug at each 24-hour time-point investigated in the hepatoma cell line PLC/PRF/5, and in fresh hepatocytes from donor number 4. A similar pattern was observed with hepatocytes from the other donors and with the other cell lines.

The assays at 24-hour intervals from the start of drug exposure showed the progressive loss of viability of treated cells compared to control cells Drug concentrations which led to 50% growth inhibition in PLC/PRF/5 decreased between 24 and 96 hours from 1 to 0.1μM for mithramycin, from >10mM to 75μM for 5-FU and from 100 μM to 1.75 μM for oxaliplatin (SRB assay). A similar decrease was

seen in fresh hepatocytes: from >10μM to 1 μM for mithramycin, from >10mM to 460 μM for 5-FU and from >100 μM to 20 μM for oxaliplatin.

The maximum cytotoxic effect of all three drugs investigated is seen 96 hours after the start of drug treatment, regardless of cell model and method of estimation. Time points beyond 96 hours were not studied, as a short assay was felt to be desirable and there was concern about maintaining fresh hepatocytes *in vitro* for longer than this timeframe. Therefore the 96 hour time point was used for the studies reported in this chapter.

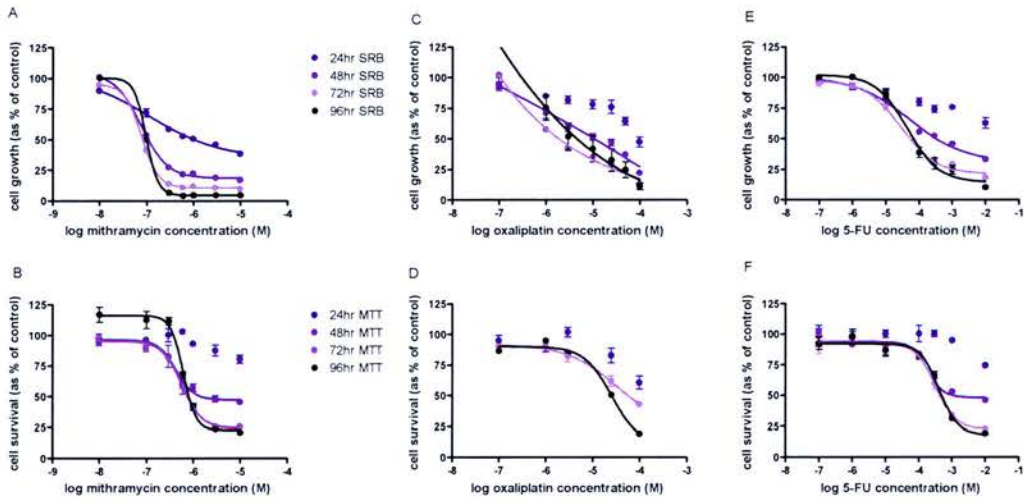


Figure 3.5: Time-course of cytotoxicity in PLC/PRF/5 and fresh hepatocytes

PLC/PRF/5 (A, C, E) and fresh hepatocytes Donor 4 (B, D, F) were exposed to a concentration range of mithramycin (A, B), Oxaliplatin (C, D), and 5-FU (E, F) for 24 hours. 24-, 48-, 72- and 96-hours after the start of drug treatment, the MTT or SRB assay was performed as described with fresh hepatocytes and PLC/PRF/5 respectively. Results are expressed as percentage of growth (PLC/PRF/5) or survival (hepatocytes) as compared to untreated cells and are the mean ± SEM of triplicate samples.

3.2.2 Comparison of SRB and MTT assays

HCT116 cells were assessed following drug treatment with oxaliplatin (figure 3.6). Cell viability (MTT) and the surviving fraction (SRB) was measured at 96 hours after the start of 24-hour drug treatment. It can be seen from figure 3.6, that the IC_{50} values obtained from the MTT and SRB assays at the 96 hour time point are

equivalent: 0.8± 0.1μM and 0.7 ± 0.1μM for MTT and SRB assays respectively. The IC_{50} values determined in this example with oxaliplatin-treated HCT 116 after 96 hours are identical, and therefore it is feasible that either assay may be used with this cell line (and the other colon cancer cell lines tested). This does not hold true for all cell types investigated but providing that the colon cancer cell lines MTT and SRB determined IC_{50}s are the same, it is valid to compare other data to only the SRB colon cancer cell line data at 96 hours.

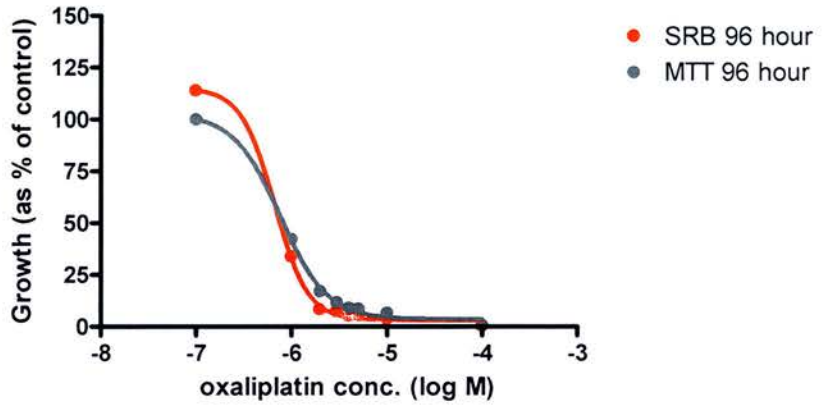


Figure 3.6: Comparison of MTT and SRB assays
1500 HCT 116 cells/well were plated 24 hours before the assay, and then treated for 24 hours with oxaliplatin. The MTT assay was performed at 24hour, 48hour, 72hour, and 96hour after the start of drug treatment and the SRB assay after 96 hours.

3.3 DRUG TREATMENT OF CELLS

The cytotoxic effect of mithramycin, oxaliplatin and 5-FU was determined in colorectal cancer cell lines and hepatocyte models (fresh hepatocytes, immortalised hepatocytes and hepatoma cell lines) to allow determination of 'active' and 'toxic' drug concentrations.

3.3.1 Cytotoxicity in Colon Cancer Cell Lines

The activity of 5-FU, oxaliplatin and mithramycin was determined in a panel of six colorectal cancer cell lines (HCT-8, HCT-15, HCT 116, HT-29, SW-620 and COLO 205) as all three drugs are active in this cancer type and allow estimation of the active concentration of these drugs.

An example of the dose response curves produced following treatment of a colorectal cancer cell line (HCT-15) with 5-FU and the SRB assay can be see in figure 3.7. The three separate curves represent 3 independent experiments carried out on separate days, making up new drug dilutions each time. Similar curves were produced for each drug and cell line. The mean of three IC_{50} values determined in independent experiments was calculated for each cell line after 24hour drug treatment and the SRB assay 96 hours after the start of drug treatment. It was verified that the IC_{50} in the colon cancer cell lines determined 96-hours after the start of 24-hour drug treatment were similar using the SRB and MTT assays. The IC_{50}s can be seen in table 3.1 and are graphically represented in figure 3.8.

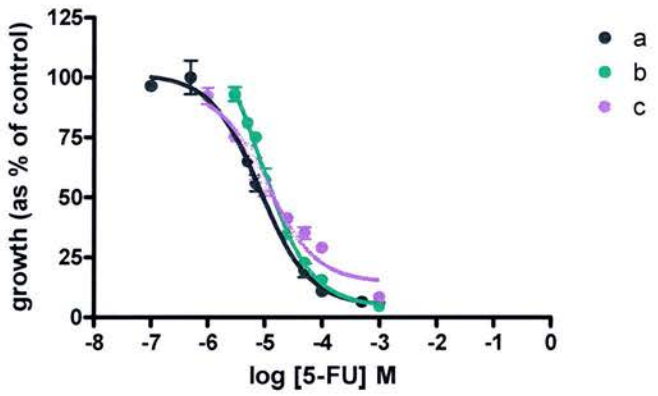


Figure 3.7: 24-hour 5-FU treatment of colon cancer cell line HCT-15
1500 HCT-15 cells/well were plated 24 hours before assay. Cells were exposed to concentrations of 5-FU ranging from 1nM to 1000μM for 24 hours, followed by SRB cytotoxicity assay 96 hours after the start of drug treatment. The experiment was carried out 3 times and a, b, and c refer to 3 separate experiments carried out on different days.

Table 3.1: Colon cancer cell line 50% inhibitory concentrations (IC_{50})
IC_{50}± standard deviation for colorectal cancer cell lines, determined 96 hours after the start of drug treatment by SRB assay in at least 3 separate experiments

	5-fluorouracil 24 hour exposure, IC_{50} (μM):	Mithramycin 24 hour exposure, IC_{50} (μM):	Oxaliplatin 24 hour exposure, IC_{50} (μM):
HCT-8	4.9 ± 2.3	0.05 ± 0.006	0.62 ± 0.16
HCT-15	12.4 ± 2.9	0.25 ± 0.006	2.35 ± 0.86
HCT 116	7.8 ± 1.2	0.03 ± 0.006	0.63 ± 0.05
HT-29	6.1 ± 2.1	0.04 ± 0	1.76 ± 0.78
SW-620	18.6 ± 4.1	0.03 ± 0	0.52 ± 0.21
COLO205	2.4 ± 0.6	0.02 ± 0.006	1.15 ± 0.16
Mean	8.7 ± 5.9	0.07 ± 0.09	1.17 ± 0.74
Median	7.0	0.035	0.89

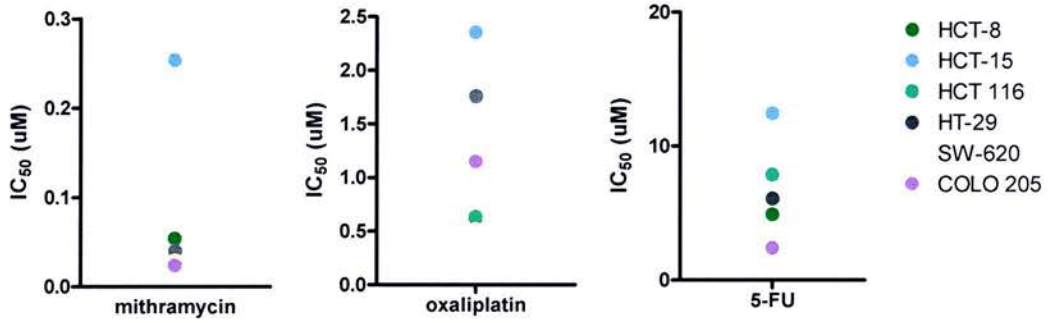


Figure 3.8: IC_{50} values in colorectal cancer cell lines
IC_{50} values were determined in 6 colorectal cancer cell lines. Cells were plated 24 hours before 24 hour drug incubation, and IC_{50} determined using the SRB assay, 96 hours after beginning drug treatment. IC_{50} values were determined from the results of at least three independent replicate experiments.

It can be seen from table 3.1 and figure 3.8 that there is variability in IC_{50} values between the different cell types treated with each of the three drugs and the potency of the 3 drugs is also very different. The cell lines were most sensitive to mithramycin with IC_{50}s ranging from 0.02 to 0.25 μM. The HCT-15 cell line is the most resistant cell line to both mithramycin and oxaliplatin, and the second most resistant to 5-FU. COLO 205 is the most sensitive cell line to both mithramycin and 5-FU. There was an 8-fold variation in IC_{50} for 5-FU across the cell lines ranging from 2.4 μM in COLO205 to 18.6 μM in SW620 cells. SW620 were the most

sensitive to oxaliplatin (IC_{50} = 0.52 μM) and HCT15 was the most resistant (IC_{50} = 2.35 μM).

For the purpose of determining a true picture of a likely IC_{50} value in the cell type in which the drugs are intended to treat, it is good to include a range of resistant and sensitive cell lines to provide a true picture. However, when the mean IC_{50} may be skewed by a particularly resistant or sensitive cell line (as was the case for mithramycin in the HCT15 cell line (Figure 3.8)) and therefore may be more appropriate to compare the toxic IC_{50} values determined in the hepatocyte models to the median IC_{50} in the colorectal cancer cell lines, as opposed to the mean.

3.3.2 Cytotoxicity in *in vitro* liver models

The cytotoxicity of mithramycin, 5-FU and oxaliplatin was next determined in different *in vitro* models of hepatocytes: 5 hepatoma cell lines, 1 immortalised hepatocyte cell line, and fresh (13)- and cryopreserved (2)- hepatocytes, with drug exposure for 24 hours in all cases. It should be noted that for the cell lines that proliferate, a dose-dependent decrease in cell growth is observed. For the fresh hepatocytes a dose-dependent decrease in cell survival is observed, as these cells do not proliferate in culture.

3.3.2.1 Cytotoxicity in hepatoma and immortalised hepatocytes cell lines

Table 3.2 and figures 3.9 show the IC_{50} values that were determined in the hepatoma cell lines (Hep 3B2.1-7, Hep G2, Huh-7D12, PLC/PRF/5, SK-HEP-1), and immortalised hepatocyte cell line (THLE-2) as determined by the SRB assay. These IC_{50}s were determined in the same way as described for the colon cancer cell lines, with mean and standard deviation calculated from 3 independent experiments.

The hepatoma cell lines showed very different sensitivity to the three drugs. Mithramycin was highly cytotoxic in all 5 cell lines with IC_{50}s ranging from 0.05 to 0.1μM. There was wider variation in the IC_{50}s for oxaliplatin and 5-FU determined in the hepatoma cell lines. The IC_{50} values ranged from 0.21 to 7.4μM for oxaliplatin and from 8 to 136 μM for 5-FU, therefore a 35- and 17-fold difference respectively.

The sensitivity of THLE-2 cells to the three drugs differed greatly. THLE-2 cells were as sensitive as hepatoma cell lines to mithramycin, and the IC_{50} values for both oxaliplatin and 5-FU were consistent with those of fresh hepatocytes and much greater than those in the hepatoma cell lines.

Table 3.2: Liver cell lines 50% inhibitory concentrations (IC_{50})

IC_{50}± standard deviation for liver cell lines, determined 96hours after the start of drug treatment by SRB assay, in at least 3 separate experiments

CELL LINE	5-fluorouracil 24 hour exposure, IC_{50} (μM):	Mithramycin 24 hour exposure, IC_{50} (μM):	Oxaliplatin 24 hour exposure, IC_{50} (μM):
Hep3B2.1-7	135.5 ± 65.8	0.08 ± 0	7.24 ± 4.19
Hep G2	8.0 ± 2.5	0.06 ± 0.01	0.21 ± 0.05
Huh-7D12	58.3 ± 7.0	0.10 ± 0.01	4.04 ± 1.96
PLC/PRF/5	75.0 ±52.3	0.10 ± 0	1.75 ± 0.91
SK-HEP-1	25.2 ± 3.25	0.05 ± 0.01	2.51 ± 0.52
Mean hepatoma IC_{50}	60.4 ± 49.6	0.08 ± 0.02	3.15 ± 2.67
Median hepatoma IC_{50}	58.3	0.08	2.51
THLE-2	234.3 ± 152.1	0.01 ± 0	11.86 ± 5.76

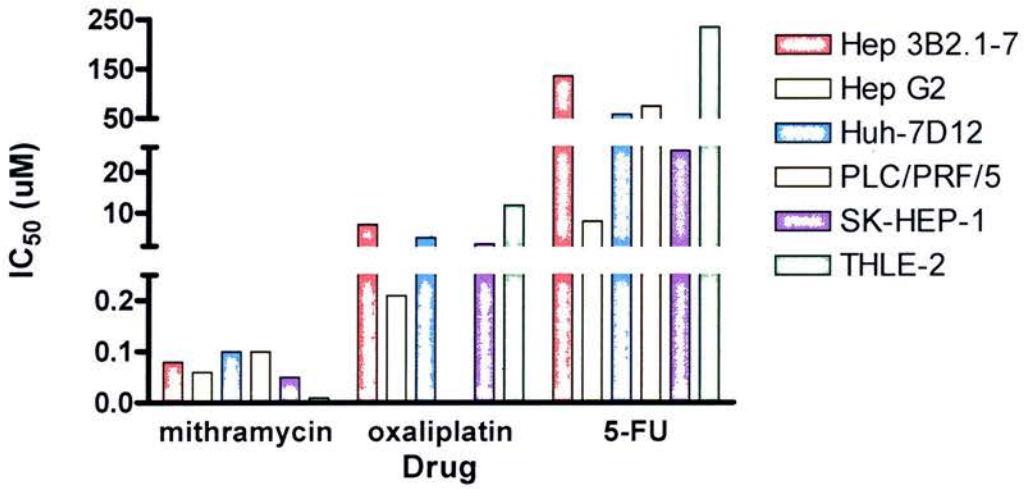


Figure 3.9: Spread of IC_{50}s in mithramycin, oxaliplatin and 5-FU–treated cell lines

IC_{50} values were determined in 5 hepatoma cell lines and the immortalised hepatocyte cell line THLE-2. Cells were plated 24 hours before 24 hour drug incubation, and IC_{50} determined using the SRB assay, 96 hours after beginning drug treatment after fitting data with a Sigmoidal dose response curve. IC_{50} values were determined from the results of at least three independent replicate experiments.

3.3.2.2 Cytotoxicity in cryopreserved hepatocytes

The sensitivity of cryopreserved hepatocytes to mithramycin, oxaliplatin and 5-FU was investigated using the MTT assay, similar to the fresh hepatocytes. The cells could only be kept in culture for a short period of time therefore surviving fractions 96 hours after the start of 24 hour drug treatment could not be detected and cytotoxicity was measured immediately following 24-hour drug treatment in the cryopreserved hepatocytes. Two drug concentrations were selected for investigation of each drug (1 and 25μM mithramycin, 25 and 250 μM oxaliplatin, and 5 and 10mM 5-FU) because the number of cells available was limited. The MTT values in control samples were much lower than usually observed in fresh hepatocytes, despite equivalent plating densities. Table 3.3 shows the surviving fraction that were determined in the cryopreserved hepatocytes at the end of 24-hour drug treatment and out of all of the drug treatments drug concentrations causing greater than 50% cell death could only be determined in hepatocytes from 1 donor. In cryopreserved hepatocytes from donor C2, the mithramycin IC_{50} was less than 25 μM and the oxaliplatin IC_{50} was less than 250μM. For all of the other donors and drugs IC_{50} concentrations were not reached.

Table 3.3: Cryopreserved hepatocyte cytotoxicity

Cytotoxicity was determined in cryopreserved hepatocytes, 24 hours after the start of 24-hour drug treatment, by MTT assay. The percentage of surviving cells at 2 drug concentrations per drug was calculated as OD as % of control cells' OD, and surviving cell percentage presented per drug concentration below.

Drug	Drug concentration (μM)	C2 #5460	C3 #5514	C4 #5654
5-fluorouracil	5000	85	104	93
	10000	61	87	83
Mithramycin	1	59	95	78
	25	37	80	54
Oxaliplatin	25	75	105	63
	250	38	92	60

3.3.2.3 Cytotoxicity in fresh hepatocytes

Table 3.4 and figures 3.10 and 3.11 show the IC_{50} values that were determined in the fresh hepatocytes (donors 1-13), as determined by the MTT assay. Fresh hepatocytes, like the hepatoma cell lines, show similar sensitivity to mithramycin

with IC_{50} values between 0.3 and 1.5μM, with the exception of hepatocytes from donor 11 which had a very high IC_{50} (>10μM). Minor variability was observed for oxaliplatin, but at IC_{50} values much greater than for mithramycin (13 to >100μM). 5-FU was the most variable among patients but also the least cytotoxic: hepatocytes from 2/13 donors were very resistant (IC_{50} >10mM), and from 9/13 donors the concentration of 5-FU that caused 50% inhibition of cell growth was between 1mM and 10mM (mean in 11/13 was 1.75mM).

Table 3.4: Fresh hepatocytes 50% inhibitory concentrations (IC_{50})

IC_{50} values for fresh hepatocytes, determined 96 hours after the start of drug treatment by MTT assay. Results are mean of triplicate samples

Hepatocyte Donor Number	5-fluorouracil 24 hour exposure, IC_{50} (μM):	Mithramycin 24 hour exposure, IC_{50} (μM):	Oxaliplatin 24 hour exposure, IC_{50} (μM):
1	269.2	0.66	19.5
2	1548.8	0.50	27.5
3	4265.8	0.93	57.5
4	457.1	0.91	19.1
5	1995.3	1.17	39.8
6	1445.4	0.38	13.4
7	1148.2	0.55	15.1
8	2570.4	1.48	>100
9	>10000	1.55	47.9
10	1995.3	0.78	30.2
11	>10000	>10	45.7
12	2570.4	1.39	44.7
13	1148.2	1.08	32.4

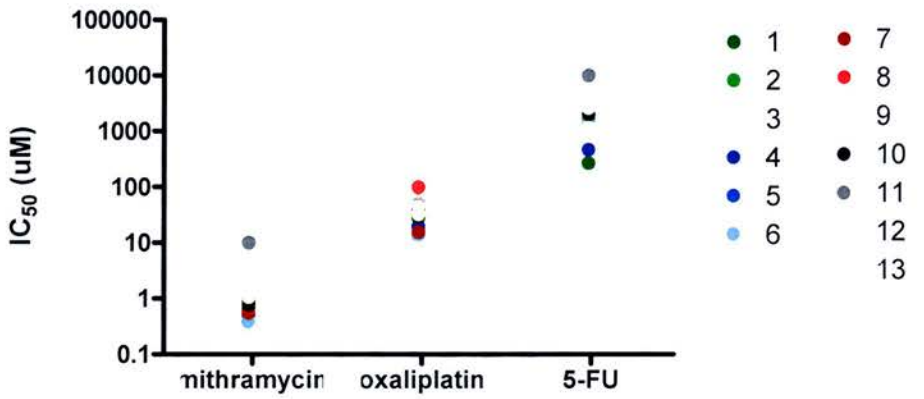


Figure 3.10: IC_{50}s in mithramycin, oxaliplatin and 5-FU–treated hepatocytes

IC_{50} values were determined in fresh hepatocytes from 13 donors. Cells were plated 12 hours before 24 hour drug incubation, and IC_{50} determined using the MTT assay 96 hours after beginning drug treatment.

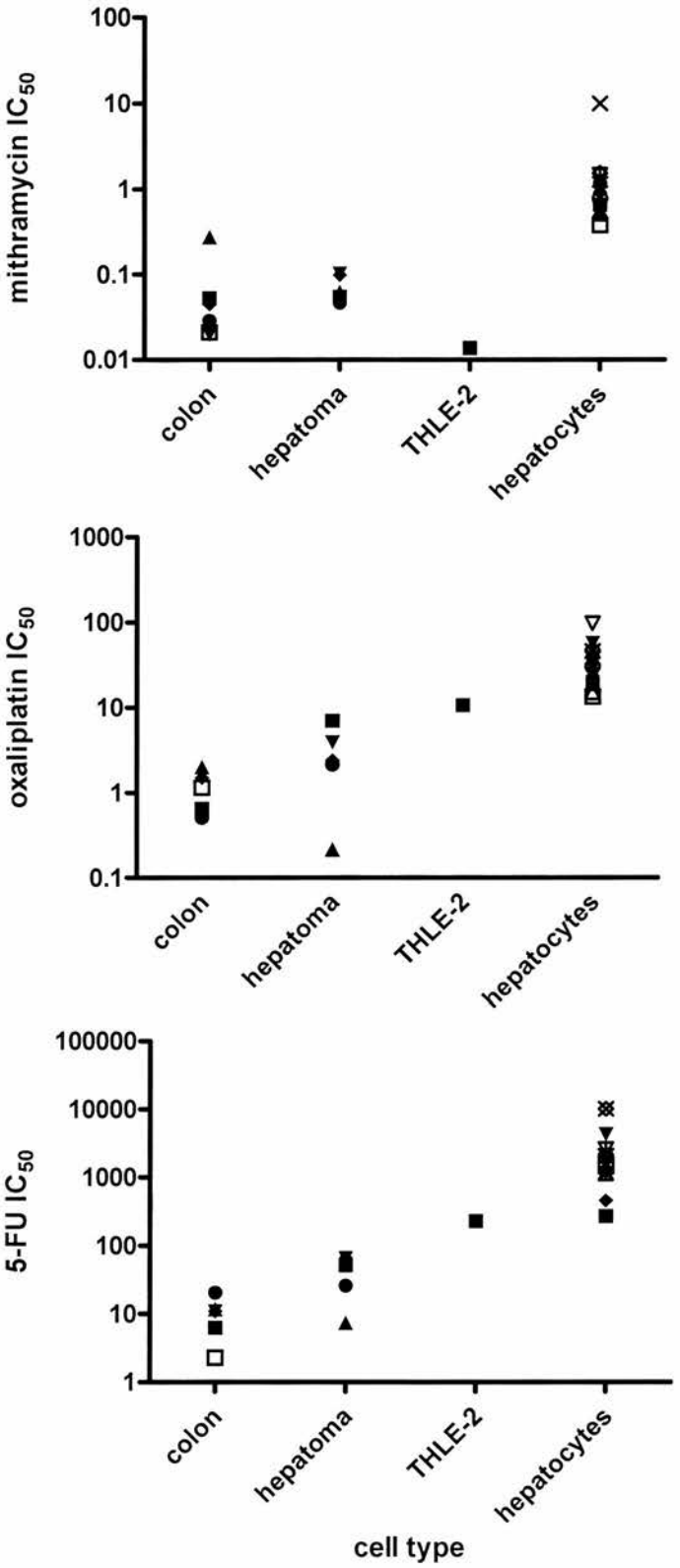


Figure 3.11: Range of IC_{50} in all cell models

IC_{50} values were determined in all models following 24-hour drug incubation, and IC_{50} determined using the MTT or SRB assay 96 hours after beginning drug treatment.

3.3.3 Therapeutic Index

The dose response curves and the IC_{50} values provide information regarding the cytotoxicity of mithramycin, oxaliplatin and 5-FU but from these data in isolation it is not possible to discriminate which drugs are likely to be hepatotoxic. By comparing the drug concentration required for effect in hepatocyte models to that in colon cancer cell lines, a clearer picture may be revealed. A ratio of the IC_{50} in hepatocyte model (toxic) to the median IC_{50} in colorectal cancer cell lines (therapeutic) can provide a therapeutic index (TI). A ratio close to 1 would suggest there is little difference between active and toxic drug concentrations and the greater the ratio between liver and colon models, the greater the therapeutic margin. For cells where it was not possible to determine an IC_{50} the TI was not calculated.

Figure 3.12 shows the therapeutic indices determined in the range of models investigated and it is apparent that there are varying sensitivities of the different cell types to each drug. In the hepatoma cell lines, mithramycin had the lowest and least variable TI with values ranging from 0.7 to 1.4. Oxaliplatin had TI values between 0.18 and 6.2. Finally, 5-FU had the highest TI ranging from 0.09 to 15.6. The TIs in THLE-2 were 27, 0.14 and 10 for 5-FU, mithramycin and oxaliplatin respectively.

The TIs varied most in the fresh hepatocytes. Mithramycin again had the lowest TI ranging from 5.4 to 22.4 (donor 11 excluded because IC_{50} not reached). Oxaliplatin had higher TI values ranging from 11 to 50 (hepatocytes from donor 8 excluded because TI not reached). Finally, 5-FU had the highest TI (ranging from 31 to 490) with the exception of hepatocytes from 2 donors (9 and 11) with resistance to 5-FU at the highest drug concentration investigated and therefore it was not possible to determine the TI (>10mM).

When comparing each drug in the different models, mithramycin had the lowest TI in all cases. When using mithramycin as a reference for hepatotoxicity, the difference in median TI values with oxaliplatin and 5-FU values was greater in THLE-2 (71- and 192-fold respectively) than in fresh hepatocytes (1- and 10-fold respectively) and hepatoma cells (2- and 8-fold respectively). Mithramycin is the

most hepatotoxic of the three drugs and has the lowest therapeutic index. Therefore, this way of comparing IC_{50} may be useful in detecting hepatotoxic drugs.

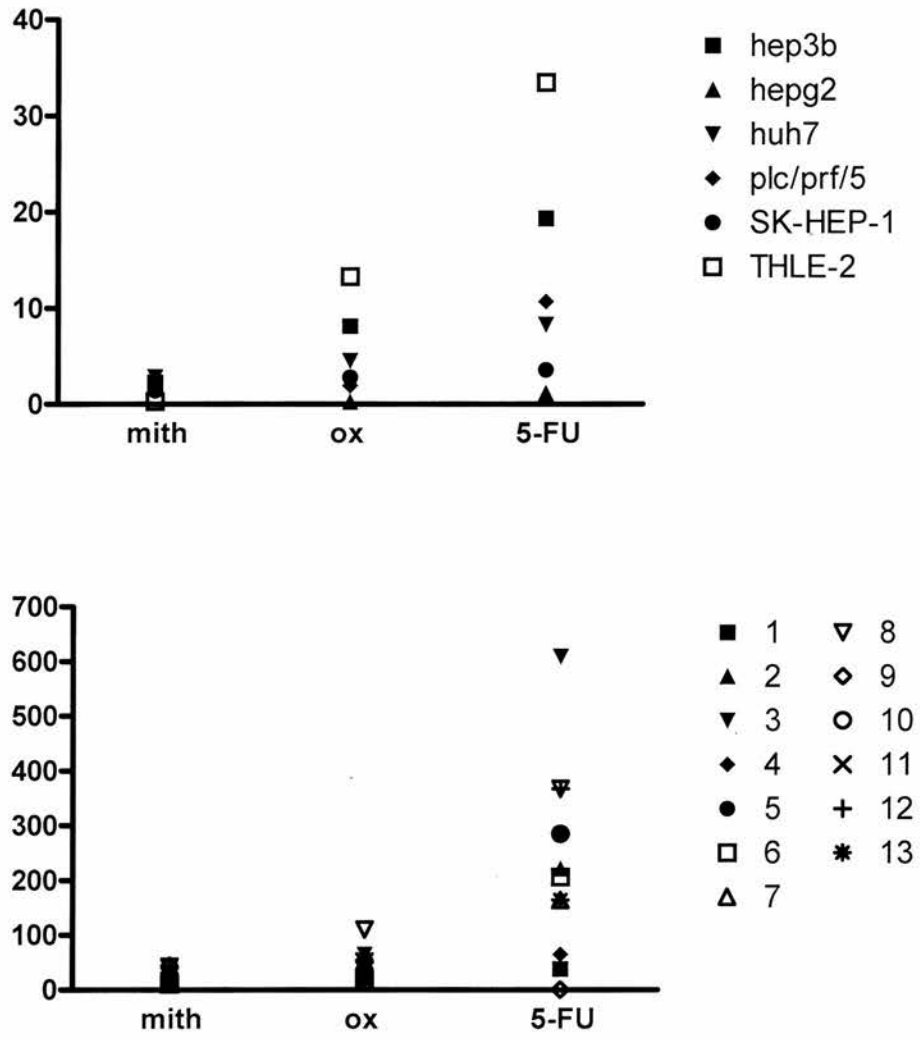


Figure 3.12: Therapeutic Index

The ratio of IC_{50} between hepatocyte models and the median IC_{50} of colorectal cancer cell lines was determined for each drug and model in cell lines (top) and fresh hepatocytes (bottom)

3.4 POTENTIAL INFLUENCES ON CYTOTOXICITY IN FRESH HEPATOCYTES

The cell lines included in this study are well established and have been used previously for several different types of investigations. However, the fresh hepatocytes that have been used for the cytotoxicity studies presented here are all from individual donors. The IC_{50} and TI values varied more in the fresh hepatocytes than in the other models tested. In order to identify potential confounding factors affecting the cytotoxicity of the drugs evaluated in fresh hepatocytes several factors were considered. The impact of hepatocyte viability and age, sex and smoking status of the hepatocyte donors on the IC_{50} for each drug was considered. Gene expression of each investigated gene at the start of drug treatment, and changes in gene expression from isolation to plating was also investigated.

3.4.1 Donor Demographics

Hepatocyte viability both at isolation and after transport were investigated. Neither donor age nor hepatocyte viability correlate significantly with the IC_{50} value determined for each drug (table 3.5). This may be considered to be a benefit, as a wide variety of donor demographics were included in this thesis, and inter-individual variability in response to drugs is to be expected. However, it does mean that these parameters do not help explain the interpatient variability in response to the drugs tested.

Table 3.5: Spearman correlation of donor factors and IC_{50} values

	Hepatocyte viability after isolation	Hepatocyte viability at arrival	Donor age
Mithramycin	r=0.0468 p=0.88 ns	r=0.1209 p=0.69 ns	r=0.1484 p=0.63 ns
Oxaliplatin	r=-0.1513 p=0.62 ns	r=-0.1154 p=0.71 ns	r=0.2637 p=0.39 ns
5-FU	r=-0.2365 p=0.44 ns	r=-0.2210 p=0.47 ns	r=-0.0470 p=0.88 ns

The effect of donor sex and donor smoking status on the IC_{50} of mithramycin, oxaliplatin and 5-FU was investigated (table 3.6). The 2 populations (male/female) do not have significantly different median IC_{50}s ($p \leq 0.01$) for any of the drugs investigated. Similarly, when comparing the IC_{50} of all drugs between smokers and non-smokers, there was no significant difference in the median IC_{50}s between smokers and non-smokers for mithramycin, oxaliplatin and 5-FU.

Table 3.6: Mann Whitney test of donor sex or smoking status and IC_{50} values
The median IC_{50}s of male vs female and smoker vs non-smoker were compared using a Mann Whitney test. The statistical significance of the difference in the medians is presented below (p-values are presented and $p \leq 0.01$ is considered to be significant)

	Donor Sex	Donor Smoking status
Mithramycin	0.20	0.60
Oxaliplatin	0.03	0.41
5-FU	0.15	0.41

3.4.2 Gene Expression

The impact of gene expression on IC_{50} was also considered. Both the change in gene expression from arrival of the hepatocytes in Edinburgh until the start of drug treatment, and the gene expression of each gene at the start of drug treatment were plotted against the IC_{50} determined for each drug for correlation analysis. Samples which did not achieve 50% growth inhibition were excluded from the analysis. Because several genes are being compared to the IC_{50}s the p-value considered to be significant has been lowered to $p<0.001$ using a Bonferroni correction because when making several comparisons the likelihood of detecting a significant correlation by chance is increased. None of the drug metabolism-related genes influenced the sensitivity to the three drugs tested.

Table 3.7 and 3.8 report the results of Spearman correlation analysis between gene expression at the start of drug treatment and change in gene expression from arrival of the hepatocytes in Edinburgh to the start of drug treatment and the IC_{50} determined for mithramycin, oxaliplatin and 5-FU respectively. No significant correlations were found.

Table 3.7: Spearman correlation of gene expression at start of drug treatment and IC_{50} values

	AAT mRNA	ALB mRNA	CYP1A1 mRNA	CYP3A4 mRNA	TTR mRNA	UGT1A* mRNA	UGT2B* mRNA	ABCB1	HIF1α mRNA	HSP-70 mRNA	P21 mRNA
Mithramycin	-0.227, p=0.50	-0.255, p=0.45	0, p=1	0.127, p=0.71	-0.282, p=0.40	0.291, p=0.39	-0.0455, p=0.89	0.1, p=0.77	-0.446, p=0.17	0.155, p=0.65	-0.127, p=0.71
Oxaliplatin	-0.350, p=0.27	-0.063, p=0.85	0.112, p=0.73	-0.049, p=0.88	-0.112, p=0.73	0.238, p=0.46	-0.021, p=0.95	0.014, p=0.97	-0.741, p=0.006	0.427, p=0.17	-0.119, p=0.72
5-FU	-0.238, p=0.51	-0.152, p=0.67	0.238, p=0.51	-0.427, p=0.22	-0.030, p=0.22	0.006, p=0.99	-0.281, p=0.43	-0.360, p=0.31	-0.512, p=0.13	0.354, p=0.32	-0.457, p=0.18

Table 3.8: Spearman correlation of change in gene expression and IC_{50} values

	AAT mRNA	ALB mRNA	CYP1A1 mRNA	CYP3A4 mRNA	TTR mRNA	UGT1A* mRNA	UGT2B* mRNA	ABCB1	HIF1α mRNA	HSP-70 mRNA	P21 mRNA
Mithramycin	0.063, p=0.85	-0.140, p=0.66	-0.203, p=0.53	-0.242, p=0.43	-0.427, p=0.17	-0.259, p=0.42	0.042, p=0.90	0.406, p=0.19	0.580, p=0.05	0.133, p=0.68	0.455, p=0.14
Oxaliplatin	0.044, p=0.89	-0.011, p=0.97	-0.269, p=0.37	-0.203, p=0.51	-0.440, p=0.13	-0.198, p=0.52	0.093, p=0.76	0.110, p=0.72	0.599, p=0.03	-0.192, p=0.53	0.308, p=0.31
5-FU	-0.050, p=0.88	0.037, p=0.91	-0.462, p=0.15	-0.151, p=0.66	-0.114, p=0.74	-0.142, p=0.68	0.101, p=0.77	0.078, p=0.82	0.348, p=0.29	-0.137, p=0.69	0.142, p=0.68

3.5 KINETICS OF CELL DEATH

The data obtained 96 hours after the start of 24-hour drug treatment has been analysed and used to determine IC_{50} values and subsequently TIs. Additionally, cell death over time was investigated in fresh hepatocytes and hepatoma cell lines. The kinetics of cell death was investigated to attempt to provide further insight into which drugs may be toxic to the liver. It was hypothesised that there may be a difference in kinetics of cell death between drugs that are intrinsically hepatotoxic and those that are not, with anti-cancer drugs that cause liver toxicity in humans causing cell death sooner than a non-hepatotoxic drug in an *in vitro* model of the liver. The loss of cell viability over time may be an indicator of the hepatotoxic potential of a drug, and cell viability was measured 24-, 48, 72- and 96-hours after the start of 24-hour drug treatment.

In order to assess progressive cell death over time one drug concentration was chosen for investigation of each cell type. A drug concentration was selected that resulted in approximately the IC_{50} value in the majority of cell samples 96-hours after the start of drug treatment (table 3.9). The percentage of surviving cells at these drug concentrations was plotted against time after drug treatment of hepatoma cell lines (figure 3.13) and fresh hepatocytes (figure 3.14). There is progressive loss of cell viability with increasing time after drug treatment in all cells. No pattern between the assays or drugs was detected for example in cells of the same type or with one particular drug there was not consistency with when the maximal cytotoxic effect was seen.

Table 3.9: Selection of drug concentration for investigation of kinetics of cell death

CELL TYPE	DRUG	MEAN IC_{50} AT 96 HOURS	MEDIAN IC_{50} AT 96 HOURS	CONCENTRATION CHOSEN
Fresh hepatocytes	Mithramycin	0.95 µM	0.91 µM	1 µM
	Oxaliplatin	33 µM	31 µM	50 µM
	5-FU	1.74mM	1.50 mM	1 mM
Hepatoma cell lines	Mithramycin	0.08 µM	0.08 µM	0.1 µM
	Oxaliplatin	3.05 µM	2.51 µM	3 µM
	5-FU	60.4 µM	58.3 µM	100 µM

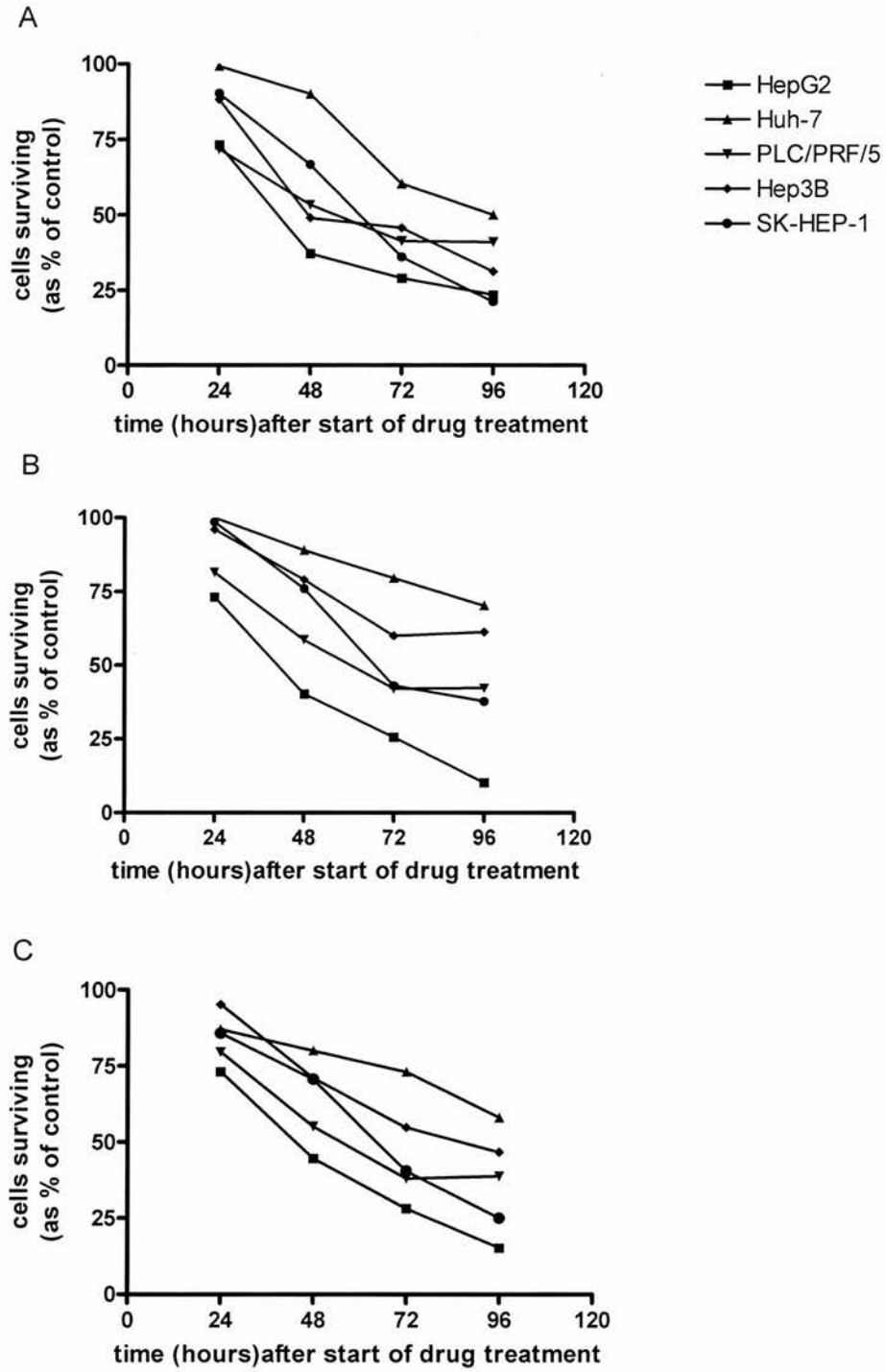


Figure 3.13: Kinetics of cell death in hepatoma cell lines after drug treatment

HepG2, Huh-7, PLC/PRF/5, Hep3B and SK-HEP-1 cells were treated with 0.1 μM Mithramycin (A), 3 μM Oxaliplatin (B) and 100 μM 5-FU (C) for 24-hours followed by the SRB assay 24-, 48-, 72- and 96-hours after the start of drug treatment. The percentage of surviving cells compared to control is plotted against time.

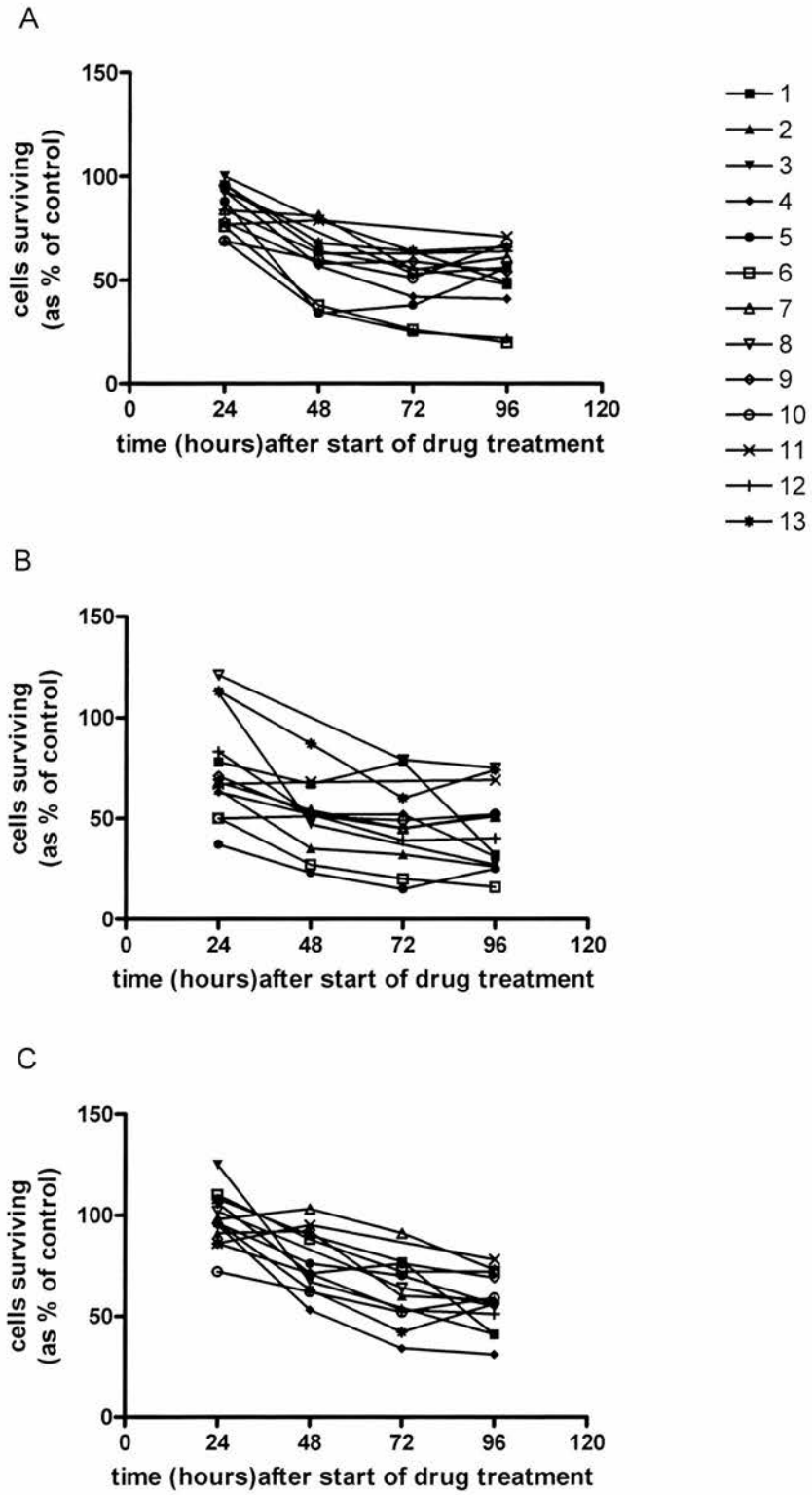


Figure 3.14: Kinetics of cell death in fresh hepatocytes after drug treatment

Fresh hepatocytes from 13 donors were treated with 1 μM Mithramycin (A), 50 μM Oxaliplatin (B) and 1 mM 5-FU (C) for 24-hours followed by the MTT assay 24-, 48-, 72- and 96-hours after the start of drug treatment. The percentage of surviving cells compared to control is plotted against time.

3.6 APPLICATION OF *IN VITRO* HEPATOTOXICITY TESTING TO DRUGS IN DEVELOPMENT

So far in these studies, it has been demonstrated that hepatocyte models are more sensitive to the hepatotoxic drug mithramycin and are less sensitive to oxaliplatin and 5-FU than the colon cancer cell lines. The therapeutic index defined for mithramycin could perhaps be used as a reference to study novel agents before they enter clinical development. New anti-cancer agents that have caused liver toxicity in pre-clinical animal models include the ruthenium compounds RM175 and HC11 (Guichard et al, 2006), and these compounds continue to be studied in clinical development.

3.6.1 Ruthenium Compounds: HC11 and RM175

In order to further test the *in vitro* models and parameters investigated so far in this thesis, RM175 & HC11 were investigated. Cells were exposed over a concentration range of both drugs for 24 hours. 24-, 48-, 72-, and 96-hours after the start of drug treatment, the MTT or SRB assay was performed (figures 3.15 and 3.16).

Both ruthenium compounds induced a rapid cytotoxic effect, detectable at the end of drug exposure (24 hours). IC_{50} values for the two ruthenium compounds were markedly different, with HC11 being 40-fold more potent (RM175 IC_{50} = 24μm, HC11 IC_{50} = 0.6μm) in the fresh hepatocytes (Donor 5).

The A549 small cell lung cancer cell line was sensitive to both RM175 and HC11. The IC_{50} values were 3 and 0.5μM for RM175 and HC11 respectively (Guichard et al, 2006) in fresh hepatocytes. The therapeutic index between A549 small cell lung cancer cell line (activity) and fresh hepatocytes are low: 9 for RM175 and 1 for HC11. In comparison, mithramycin has a TI of 11 in this donor suggesting that both RM175 and HC11 might induce hepatocyte toxicity (figure 3.17). HC11 was found to be more hepatotoxic than RM175 in *in vivo* mouse models (Guichard et al, 2006) and this pattern is confirmed *in vitro* with both fresh hepatocytes and hepatoma cell lines, strengthening the *in vivo* data and providing more confidence in the *in vitro* model.

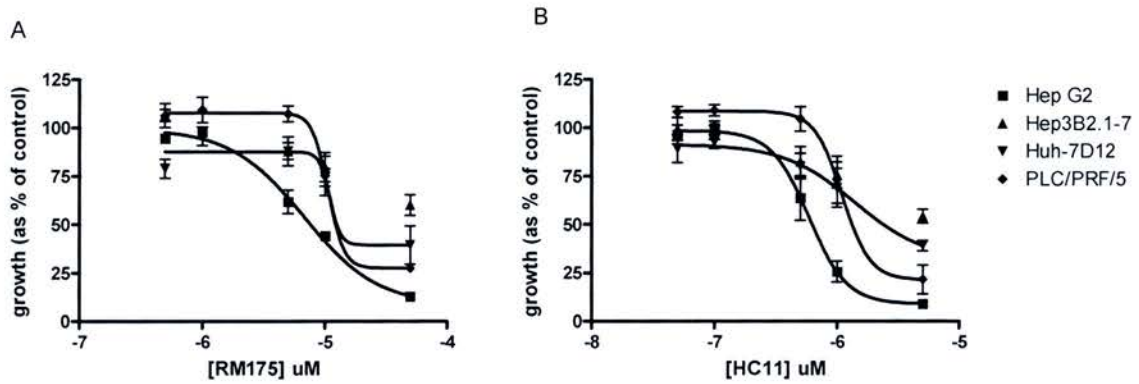


Figure 3.15: Cytotoxicity of RM175 (A) and HC11 (B) in 4 hepatoma cell lines using the SRB assay 96-hours after the start of drug treatment

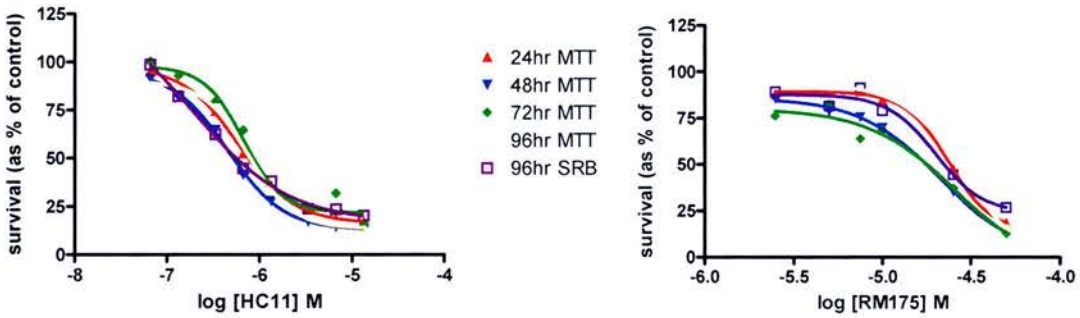


Figure 3.16: Cytotoxicity of HC11 (left) and RM175 (right) in fresh hepatocytes DONOR 5

Cells were treated over a concentration range for 24 hours. 24-, 48-, 72- and 96-hours after the start of drug treatment, the MTT assay was performed as described, and at 96-hours the SRB assay as described. Results are expressed as percentage of survival as compared to untreated cells and are the mean of triplicate samples.

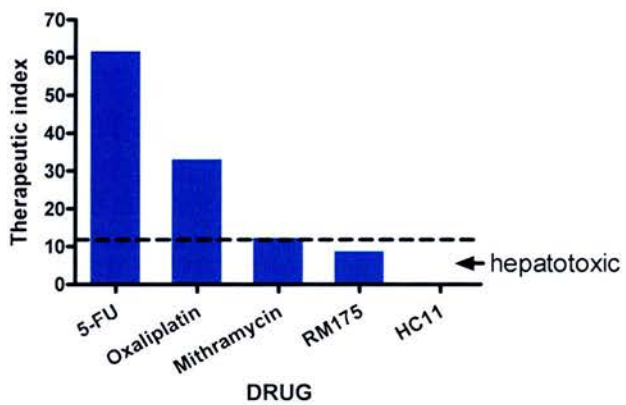


Figure 3.17: Therapeutic index of 5 anti-cancer agents

Using IC_{50} concentrations determined in fresh human hepatocytes (donor 5) and in non-small cell lung cancer cell line A549, TIs were calculated for 5-FU, oxaliplatin, mithramycin, RM175 and HC11. The dotted line represents the TI of mithramycin, which may be used as a reference below which a drug could be considered hepatotoxic. The TIs for RM175 and HC11 were lower than that of mithramycin.

3.7 DISCUSSION

In vitro models are used to investigate liver toxicity with many classes of drugs. As highlighted earlier, the differences between anticancer drug mode of action, combined with the fact that the liver is a non-replicating organ, necessitates that the study of anti-cancer drug induced liver toxicity requires special consideration. The selection of drugs with characterised effects in humans has aided the testing of the suitability of the *in vitro* models for detecting liver toxicity.

The concept of accurate prediction of anticancer-drug induced toxicity using *in vitro* models has been investigated for the detection of other toxicities, for example myelotoxicity, caused by anti-cancer drugs. The colony-forming unit granulocyte macrophage (CFU-GM) assay has been developed for the evaluation of human myelotoxicity, defining IC_{50}, IC_{75} and IC_{90} values (Masubuchi et al, 2004). Using human and murine IC_{90} values for myelotoxicity of the investigated compounds (5 campothecin derivatives) in combination with other tests, the human MTDs were predicted retrospectively (Masubuchi et al, 2004) and it was suggested by the authors of this paper and others (Malerba et al, 2004, Pessina et al, 2003) that the CFU-GM *in vitro* model could be used in the investigation of toxicity during the development of new anti-cancer agents. This example shows that it is possible to develop *in vitro* assays to predict specific anticancer drug toxicities, but they require thorough validation. There is no published validated model routinely used for the prediction of liver toxicity with anti-cancer agents, and the investigation of this is the purpose of this thesis.

3.7.1 *In vitro* cytotoxicity assays

The two assays selected were the MTT and SRB assays and both are suitable for use with cancer cell lines and hepatocytes. The SRB assay is an established technique used by the National Cancer Institute (NCI) for cytotoxicity studies with cancer cell lines (http://dtp.nci.nih.gov) and there are many published papers that present results from the SRB assay. The MTT assay is used by Celsis (formerly Invitrotech) with human hepatocytes (http://www.celsis.com), and again there are many published papers using the MTT assays with cell lines.

Several other models have been used for investigation of hepatotoxicity with other classes of drugs, and there are no publications suggesting that an accurate model for prediction of anti-cancer drug induced hepatotoxicity has been thoroughly investigated or determined. Examples of published research using different assays and model systems are provided below.

Biagini et al (2006) performed *in vitro* MTT and ATP assays using liverbeads® (cryopreserved hepatocytes entrapped in alginate beads) and WIF-B9 (hybrid cell line obtained by fusion of rat hepatoma [Fao] and human fibroblasts [WI38]) to investigate hepatotoxicity. They used several reference hepatotoxic compounds, none of which were anti-cancer agents, and found that both models could be useful for investigating the hepatotoxic potential of the selected chemicals using MTT and ATP assays.

Kikkawa et al (2005) investigated hepatotoxicity using LDH release and WST-1 assays in rat hepatocytes with reference hepatotoxic compounds, and found that the WST-1 assay that assesses mitochondrial respiration detected cytotoxicity earlier than the LDH release assay with the same compounds, and therefore concluded that an assay that detects mitochondrial function was useful for predicting hepatotoxicity.

Harries et al (2001) investigated the use of genomics technology to investigate gene expression changes associated with hepatotoxicity in Hep G2 cells following exposure to carbon tetrachloride and ethanol. Microarray mRNA analysis revealed that different groups of genes were up-/down-regulated by ethanol or carbon tetrachloride and suggested that different mechanisms of hepatotoxicity may be associated with particular patterns of gene expression, however further investigations into this would be required. Similarly, HepG2 cells were used by Burczynski et al, 2000 to perform microarray analysis in cells treated with cisplatin and non-steroidal anti-inflammatory drugs. It was concluded that whilst the results from HepG2 may be useful in some settings Burczynski et al were going to investigate other biological

systems that could yield more reproducible and robust transcriptional profiles than HepG2 cells, however to date they have not published any similar work.

A similar approach to that used in the studies presented in this thesis was employed by Li et al (1999) using cryopreserved hepatocytes with the MTT assay and they found this combination to be a useful model for predicting hepatotoxicity with known hepatotoxic compounds.

The above examples demonstrate that several model systems have been investigated for the study of hepatotoxicity. For this thesis, it was decided to select assays that would assess different stages of cell death for the assessment of hepatotoxicity. The MTT assay detects mitochondrial function whereas the SRB assay detects the total protein. Using the MTT and SRB assay in all colon cancer cell lines, no differences were detected in the IC_{50}s 96-hours after the start of drug treatment between the assays. However, in the fresh hepatocytes and the other cell lines there were differences between IC_{50}s determined using MTT and SRB after 96hours, but a donor- or drug-specific pattern of which assay was more sensitive was not revealed by examining IC_{50}s at 96-hours for the three drugs from all 13 fresh hepatocyte-donors.

3.7.2 Drugs tested and Therapeutic Index

5-FU and oxaliplatin are used in the clinic for treatment of patients with colorectal cancer. Mithramycin is infrequently used to treat patients due to the liver toxicity it causes, however from the NCI60 panel of cell lines, the sensitivity of colorectal cell lines (used in this thesis) and several others in which mithramycin has been used in the clinical setting (e.g. testicular, leukaemic) is equivalent (http://dtp.nci.nih.gov/).

The cell models were treated over a range of drug concentrations. It was of interest to compare the IC_{50} concentrations determined *in vitro* to concentrations of drug achievable in patients. The dose of anti-cancer agents administered to patients is calculated based on their body surface area, to allow the maximum dose for efficacy and minimising toxicity in different patients. Pharmacokinetic studies allow

detection of drug concentration of administered drugs in humans. 5-FU concentrations have been reported in humans (n=39) following oral treatment with 5`-DFUR for at least 5 consecutive days at a mean total dosage per patient of 6.4g, with blood and liver samples collected 3-5 hours after final administration (Zheng and Wang, 2005). The mean plasma and liver 5-FU concentrations were 8.5μM and 43μM respectively (Zheng and Wang, 2005). Another study by Findlay et al (1996) demonstrated that plasma 5-FU concentrations following administration of 5-FU on a protracted venous infusion for 8 weeks (300mg/m^2/day) ranged from less than 0.192μM to 192μM maximum over the 8 weeks, and commented that these plasma concentrations were higher than those previously reported where steady state plasma 5-FU levels were up to 1.5μM in the first 48 hours of infusion. Therefore the IC_{50} drug concentrations causing cytotoxicity *in vitro* for 5-FU in the cells lines in this thesis are equivalent to drug concentrations achievable in humans, and for the fresh hepatocytes from the majority of donors, 5-FU concentrations achievable in humans are lower than those required to cause 50% inhibition of cell survival of hepatocytes *in vitro*. The concentration of 5-FU required to cause hepatocyte cell death *in vitro* are high and therefore the concept that dose determines poison is relevant here, given that 5-FU is not widely hepatotoxic in patients. Oxaliplatin concentrations in human plasma following a single 1-4 hour infusion of 130mg/m^2 oxaliplatin were reported to range from 3.8μM to 8.1μM in a review of 7 studies on oxaliplatin pharmacokinetics published by Graham et al (2000). Similar to 5-FU, the concentration of oxaliplatin achievable in patients is similar to the IC_{50}s determined in the cell lines and lower than those determined in the fresh hepatocytes in this thesis. A thorough literature review revealed no human pharmacokinetic studies of mithramycin, perhaps due to its limited clinical use; therefore comparison with concentrations achievable in patients is not possible. It should be noted though that again the IC_{50}s for mithramycin determined in the cell lines is approximately 10-fold lower than those in hepatocytes, and the molar IC_{50}s for mithramycin are lower than for oxaliplatin or 5-FU. The type of experiments presented in this thesis may be used to provide an idea of risk with novel agents at concentrations beyond those used in the clinic. Knowing the concentrations of drugs achievable in patients it would be of interest to see how many donors would reach loss of viability at clinically achievable

concentrations, however with these data not being available for mithramycin, this could not be investigated.

The drugs selected (mithramycin, oxaliplatin and 5-FU) do not require cytochrome P450 enzymes for their metabolic activation or detoxification, although 5-FU is metabolised by other enzymes in the liver and it is likely that these are differentially expressed between hepatocyte donors and cell lines. A preclinical study from our laboratory recently showed that liver gene expression can inform the drug metabolism of capecitabine, a 5-FU pro-drug (Guichard et al, 2007). Whilst the metabolism of 5-FU was not extensively studied in this paper, an enzyme involved in 5-FU catabolism was evaluated and dihydropyrimidine dehydrogenase expression was found to be higher in human liver than in HCT116 xenografts in mice. Evaluation of enzymes involved in metabolism of 5-FU and other drugs may provide further insight into hepatotoxic potential of drugs, but would have to be considered on a case-by-case basis. Oxaliplatin was selected as a negative control when these studies began, however there is an increasing body of evidence that oxaliplatin can cause liver toxicity. The studies presented in this thesis found oxaliplatin to have a higher therapeutic index than mithramycin in all groups of cell models, agreeing with clinical study suggesting that oxaliplatin is not acutely toxic to hepatocytes. Mithramycin has been infrequently used due to the liver toxicity it can cause, however more recently the use of mithramycin in combination with bevacizumab has been investigated (Jia et al, 2007). Duverger et al (2004) found that mithramycin A sensitises tumours cells to apoptosis induced by tumour necrosis factor (TNF). These examples highlight the issue that increased knowledge could lead to increased use and adaptive dosing strategies for example in combination at lower doses of drugs, or prolonged administration to avoid peak concentrations, when knowledge of their hepatotoxic potential is obtained.

The data presented in these studies assess acute toxicity in that a single short exposure of drug was used. The majority of 'classical' cytotoxic anticancer agents are administered cyclically where a period of recovery between each dose is allowed. Perhaps the study of acute toxicity and not long-term toxicity is indeed more relevant

with anticancer drugs because cytotoxic anti-cancer drugs are used cyclically and the body is allowed to recover in between, and generally these drugs are only used over a period of a few months. The experimental design of the studies presented herein is therefore relevant for cytotoxics.

The IC_{50} concentration was chosen to compare the two models as it is a commonly accepted method for reporting cytotoxicity caused by anticancer agents. The maximum cytotoxic effect reached using the chosen concentration ranges was not 100% in most cell models and to achieve complete cell kill exceptionally high drug concentrations may have been required, that would be irrelevant *in vivo*. Also the aim was to detect clinically significant toxicity and this will occur at doses lower than those required to induce complete hepatocyte cell death. At very low drug concentrations it has been observed that in some cases an increase in viable cells is seen before any decrease and therefore determining a small percentage of cell death (at lower drug concentrations) may be more difficult and less informative.

The concept of defining a therapeutic index is used for several classes of drugs, not solely anticancer agents. Another example is antiretroviral agents for which the ratio of efficacy to toxicity *in vitro* is often defined to provide information of a drugs potential clinical utility (Buss and Cammack, 2001). The therapeutic index defined for mithramycin, oxaliplatin and 5-FU by comparing cytotoxic concentrations in hepatocyte models and colon cancer cell lines revealed differences between the known hepatotoxin mithramycin and the other two drugs (5-FU and oxaliplatin). The use of a therapeutic index as defined in these studies has the potential to increase drug safety. Currently, the development of anti-cancer agents will assess the active concentrations of the new compound against a range of cancer cell lines derived from different cancer types. The use of human liver is often limited to the exploration of metabolism with short term studies in either intact cells or microsomes. The studies presented here have demonstrated that, for cytotoxic agents, the use of hepatocytes over 96 hours using cell viability as the endpoint provides more information as to the hepatotoxic potential of a compound. Furthermore, the calculation of the TI provides

insight into the likelihood of achieving active concentrations of the compounds without hepatic side effects.

The approach of defining a TI was useful when investigating the novel anticancer agents RM175 and HC11. Preclinical data obtained in mice indicated potential liver toxicity after treatment with HC11, and to a lesser extent with RM175. When tested *in vitro* HC11 was found to be more hepatotoxic than RM175 in fresh hepatocytes, confirming pre-clinical rodent data. This finding further alerted to the hepatotoxic potential of HC11 for its clinical development, and provided more confidence in the *in vitro* models for detecting hepatotoxicity.

In summary, defining the therapeutic index of the investigated drugs revealed a wide range of therapeutic indices in the different models. It is promising that the known hepatotoxic agent mithramycin has lower TI than with 5-FU or oxaliplatin and that the more hepatotoxic of the novel agents investigated, HC11, had a lower TI than the other compound investigated.

3.7.3 Influences on Cytotoxicity in Fresh Hepatocytes

Several parameters were investigated that could impact on cytotoxicity in the fresh hepatocytes, and these included hepatocyte viability, donor sex, age and smoking status, and gene expression of selected genes. No donor factors were found to associate with the IC_{50} values. The impact of gene expression on hepatocytes IC_{50}s for each drug was also considered and of the investigated genes, none were found to correlate with the IC_{50}s for any of the investigated drugs.

3.7.4 Time-points selected and Kinetics of cell death

The time-points of 24-, 48-, 72- and 96- hours after the start of 24-hour drug treatment were selected to allow detection of progressive toxicity. The time-course of the assays selected was based on the optimum duration of culture for the hepatocytes and is also the length of assay used by the NCI for the SRB assay (based on average doubling time of their panel of 60 cancer cell lines).

It was hypothesised that due to the different mechanisms of action of the 3 drugs tested, that differences in the kinetics of cell death would be noted, but on analysis of this in the hepatoma cell lines and in the fresh hepatocytes no pattern between the different drugs was observed. This is important because it suggests that short-term assays may not detect accurately the hepatotoxic potential of some agents. It would therefore be suggested that a 96-hour assay assessing cell viability is set up to evaluate the impact of drugs tested on *in vitro* liver models.

3.7.5 Conclusions

Based on the data presented in this chapter the majority of the *in vitro* liver models (fresh hepatocytes, THLE-2 and three of the hepatoma cell lines) can discriminate the hepatotoxic drug mithramycin from other anticancer compounds (5-FU and oxaliplatin). THLE-2 cells are more sensitive to mithramycin than the tumour cells and accurately discriminated mithramycin as hepatotoxic. THLE-2 is a single cell line that did not express significant levels of several of the liver-specific genes. Whilst it was predictive for these three drugs it may not be in other situations, therefore further exploration of the use of THLE-2 cells should be undertaken. The SK-HEP-1 cell line could not discriminate the hepatotoxic drug and are therefore unlikely to be useful. The SK-HEP-1 cell line is derived from endothelial cells and not from hepatocytes, which may help explain its lack of ability to discriminate hepatotoxic drugs. The method of determining a therapeutic index is proposed to be useful for the detection of hepatotoxic anti-cancer agents. Further work using HC11 and RM175 confirmed the possibility of detection of hepatotoxicity using fresh hepatocytes and hepatoma cell lines. However, it would be desirable to investigate more compounds to confirm these findings.

Drawing conclusions from 13 donors would generally be considered a very small sample. However, several published reports of work using human hepatocytes reports results from 4 or less donors (Baughman et al, 2005; Kostrubsky et al, 2003; Kostrubsky et al, 2005). By comparison the number of samples from which data has been generated in these studies is high. However, there is significant inter-donor

variability in response to each drug tested. Notably the least variation is seen in response to mithramycin.

The comparison of therapeutic and toxic drug concentrations to determine hepatotoxic drugs is potentially very useful and has not been reported previously. Whilst the response of the cells to the drug treatment is interesting alone, the comparison allows more meaningful conclusions to be drawn. However, the prospect of detecting cell death sooner and obtaining insight into the mechanism of cell death caused is appealing and will be investigated in the following chapter.

CHAPTER 4: Results
Earlier detection of cell stress induced by anti-cancer agents in selected *in vitro* models of the liver

4.1 INTRODUCTION
- 4.1.1 Methods Available For Detection of Cell Death
- 4.1.2 Chapter Aims and Overview

4.2 ESTABLISHMENT OF THE SELECTED *IN VITRO* ASSAYS
- 4.2.1 Adenylate Nucleotide Ratio Assay
- 4.2.2 Caspase-3/7 Assay

4.3 ATP LEVELS IN CELLS FOLLOWING DRUG TREATMENT
- 4.3.1 ATP Levels in Cells Following Mithramycin Treatment
- 4.3.2 ATP Levels in Cells Following Oxaliplatin Treatment
- 4.3.3 ATP Levels in Cells Following 5-FU treatment

4.4 CASPASE-3/7 ACTIVATION IN CELLS FOLLOWING DRUG TREATMENT
- 4.4.1 Caspase-3/7 Activation Following Mithramycin Treatment
- 4.4.2 Caspase-3/7 Activation Following oxaliplatin Treatment
- 4.4.3 Caspase-3/7 Activation Following 5-FU Treatment

4.5 COMPARISON OF EARLY (24-HOUR) AND LATER (96-HOUR) DATA
- 4.5.1 Mithramycin
- 4.5.2 Oxaliplatin
- 4.5.3 5-FU

4.6 DISCUSSION
- 4.6.1 Adenylate Nucleotide Ratio Assay and Results
- 4.6.2 Caspase-3/7 Assay and Results
- 4.6.3 Mechanisms of Cell Death in Hepatocyte Models
- 4.6.4 Earlier Detection of Cell Death
- 4.6.5 Conclusions

4.1 INTRODUCTION

In the previous chapter, drug-induced hepatocyte toxicity was detected using cytotoxicity assays; therefore cell death was considered as the endpoint. However, it would be desirable to detect cell stress induced by anti-cancer agents earlier following drug treatment. As outlined in section 1.2.2, cells die via two main mechanisms, namely apoptosis and oncosis. The possibility of finding an assay to detect oncosis or apoptosis and provide insight into the mechanism of cell death resulting in toxicity, and to identify liver toxicity sooner than the cytotoxicity assays presented in chapter 3, was investigated.

Different mechanisms of cell death have been identified following anti-cancer drug treatment, based on biochemical and morphological features within cells. Notably, drugs have different biochemical targets, e.g. platinum compounds target DNA, and taxanes target microtubules (see section 3.1.2 for mechanism of action of drugs used in this thesis), but all drugs ultimately kill cells by activating biochemical cascades of apoptosis or by causing oncosis. Research until the end of the 1980s suggested that anti-cancer drugs induced cell death by a non-specific and uncontrolled oncotic mechanism (Martin et al, 2000). However, a greater understanding of apoptosis from 1990 onwards and many investigations involving anticancer agents, both *in vitro* and *in vivo*, have proved the case for the involvement of apoptosis in tumour-cell death following administration of several classes of anticancer drugs (Fulda et al, 2001; Kim et al, 2002; Petak and Houghton, 2001). For example, 5-FU has been shown to induce apoptosis in many cancer types including salivary gland cancer cell lines, colon cancer cell lines and in liver metastases of colorectal cancer patients (Aota et al, 2000; Backus et al, 2001).

There is now clear evidence that apoptosis and oncosis are not mutually exclusive processes (Fink and Cookson, 2005; Jaeschke and Lemasters, 2003), but rather that a combination of both may occur in different cells. The complexity of interacting processes in dying cells occurring simultaneously should be acknowledged. Furthermore, inhibiting one part of the cell death signalling pathways may not result in cell survival and may solely act to execute another pathway (Nicotera et al, 1998).

Leist et al, 1997 provide evidence that cells triggered to undergo apoptosis are instead forced to die by necrosis when ATP energy levels are rapidly compromised. The mechanism of cell death can also vary, depending on the drug in question, the drug concentration and which model is being used to study it.

4.1.1 Methods Available for Detection of Cell Death

Several methods are available for the detection of apoptosis and oncosis *in vitro* (see figure 4.1). Apoptosis involves a cascade of events occurring sequentially, and methods are available to detect both early and late stages of apoptosis. The characteristics of apoptosis are the same regardless of how apoptosis is initiated: permeabilisation of the mitochondrial membrane; the appearance of phosphatidylserine at the plasma membrane; chromatin condensation and fragmentation in the nucleus; and caspase activation (Lawen, 2003), and methods are available for detecting these different stages. Characteristics of oncosis are less well defined than for apoptosis, however rapid permeabilisation of the plasma membrane and leakage of intracellular contents occur during oncosis and there are methods available for the detection of these parameters.

Plasma membrane permeability may be studied with a variety of techniques including propidium iodide (PI) and annexin V (AV) staining, and ATP and LDH depletion or leakage. Living cells with an intact plasma membrane exclude cationic dyes such as PI or trypan blue under normal circumstances. When the cell membrane becomes damaged, these dyes can enter the cell. Apoptotic cells will maintain the integrity of the cell membrane for longer than oncotic cells, which would be very quickly stained by dyes such as PI due to extensive membrane damage. Analysis of PI positive and negative cells is usually performed by flow cytometry and is not commonly used with adherent cells: trypsinisation of adherent cells would be required to generate a cell suspension which can damage the cell membrane and lead to artificial PI-positivity.

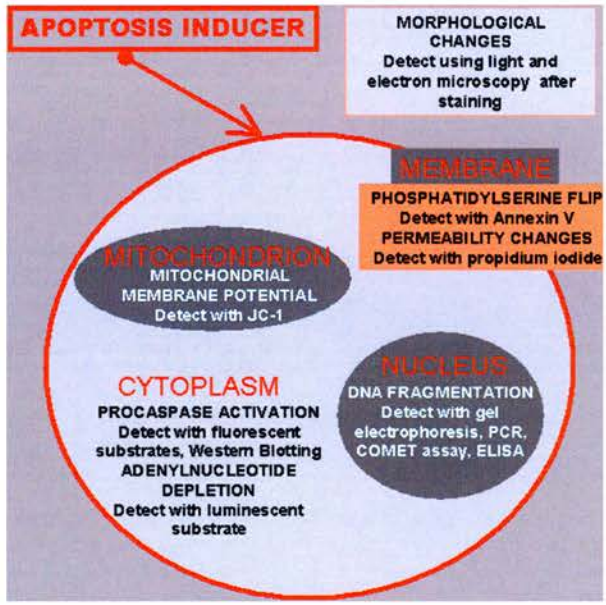


Figure 4.1: Summary of methods available for detection of apoptosis or oncosis

Several methods are available for the detection of apoptosis and oncosis. Morphological and biochemical changes occur and their cellular location is demonstrated.

Early during apoptosis, phospatidylserine (PS) normally present in the inner layer of the cell membrane, flips to the outer membrane. Annexin V readily binds to PS, and conjugates of annexin V with a fluorescent dye such as fluorescein isothiocyanate (FITC), are often used to detect cells with exposed PS. As with PI detection, analysis of Annexin V-positive cells is performed using flow cytometry, meaning that this technique is not ideal for adherent cells. If plasma membrane damage is extensive, annexin V will gain access to and bind PS present in the inner membrane of cells. It is therefore recommended to perform dual staining with both annexin V and PI to discriminate apoptotic (AV+/PI-) from oncotic (AV+/PI+) cells.

Membrane damage during oncosis results in leakage of molecules from the cell *in vitro* into the cell culture medium. Small molecules such as ATP leak rapidly, and rapid and extensive loss of ATP is a marker of oncosis. Apoptosis is an energy requiring process, however cells undergoing apoptosis will maintain higher levels of ATP for a longer period of time than those cells dying by oncosis. It has been reported that the ratio of ADP:ATP can be used to discriminate between cell death

occurring via apoptosis and what is referred to as oncosis in *in vitro* systems (Bradbury et al, 2000). Another cellular macromolecule, which can be studied to assess cell death, is the cytoplasmic enzyme lactate dehydrogenase (LDH). Following loss of plasma membrane integrity during apoptosis or oncosis, LDH will exit the cells and colorimetric assays are available to detect LDH in tissue culture medium.

Caspase activity can also be detected as a marker of early-stage apoptosis. Caspase-3 is activated whether apoptosis occurs via the receptor or mitochondrial pathway (Fulda et al. 2001). Caspase-3 and caspase-7 are effector caspases rather than the initiator caspases, and therefore their activation occurs following activation of the relevant initiator caspases. Pro-caspases are cleaved during apoptosis and it is possible to detect caspase activation via Western blotting and via the use of a pro-fluorescent substrate. As mentioned previously, Western blotting is not suitable for screening purposes with many samples and the use of a pro-fluorescent substrate is more suited to this purpose. This type of caspase assay requires a labelled dye which fluoresces when cleaved by caspases. The intensity of the measured fluorescence is therefore proportional to caspase activation, and this technique is suitable for adherent cells.

Mitochondrial membrane permeability (MMP) is a relatively late event occurring during apoptosis, and is usually detected using dyes with high affinity for the mitochondria. JC-1 is a commonly used fluorescent cationic dye that accumulates in the mitochondria of healthy cells as aggregates which fluoresce red. Upon the onset of apoptosis, the mitochondrial membrane potential collapses and the JC-1 dye can no-longer accumulate and aggregate in the mitochondria and therefore remains in the cytoplasm in its monomeric form (which fluoresces green). The MMP is measured as the differential distribution of red and green fluorescence, and is usually detected by flow cytometry but analysis can also be performed by fluorescence microscopy. Cytochrome c release from the mitochondria may also be detected, usually by Western blotting. This technique is low-throughput, requires many cells and is therefore not practical for screening purposes.

Finally, DNA fragmentation is characteristic of late-stage apoptosis, and can be detected using several techniques. PI staining with flow cytometric or microscopic detection can be used to assess DNA fragmentation as PI also readily binds to DNA. Another technique used to examine DNA fragmentation during apoptosis is the terminal uridine deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. DNA fragments generated during apoptosis can react with the terminal deoxynucleotidyl transferase enzyme which catalyses the addition of dUTPs that are labelled with a marker, which allows the DNA fragmentation to be detected.

4.1.2 Chapter Aims and Overview

Understanding more about the mechanism of cell death induced by anticancer drugs in hepatocyte models may provide a better understanding of the observed toxicities in humans. The mode of cell death induced is relevant to the subsequent fate of the tissue in that apoptotic bodies are rapidly recognised and phagocytosed by either macrophages (Kupffer cells are the resident macrophages of the liver) or adjacent epithelial cells. Conversely, oncotic cell death results in cell rupture, releasing the cell contents into the surrounding tissue and will most likely result in an inflammatory response and potentially a greater area of damage in the liver.

A rapid, sensitive, specific assay was required, that can detect early rather than late consequences of cellular response to drugs, and therefore 2 techniques from those outlined in section 4.1.1 were selected. The cell models have been further investigated, following 24-hour drug exposure, using an assay that measures ATP levels in cells, and an assay that measures caspase-3/7 activity.

Four of the hepatoma cell lines (Hep3B, Huh-7D12, HepG2 and PLC/PRF/5), fresh hepatocytes from 2 donors (12 and 13), and cryopreserved hepatocytes from 3 donors (C2, C3 and C4), were exposed to 5-FU, mithramycin and oxaliplatin to evaluate whether the 2 selected assays could detect hepatocyte toxicity earlier than the cytotoxicity assays described in chapter 3. It should be noted that the number of cryopreserved hepatocytes available were limited, and therefore selected doses of

each drug were tested. The response of the models to drug treatment in terms of caspase-3/7 activation and/or ATP loss may aid in the investigation of which *in vitro* models respond in a similar way to the hepatocytes in response to particular drugs and provide insight into the mechanism of cell death occurring after drug exposure.

4.2 ESTABLISHMENT OF THE SELECTED *IN VITRO* ASSAYS

After assays were selected to measure ATP, ADP and caspase-3/7 in drug treated *in vitro* models of the liver, preliminary experiments were carried out to establish their performance and sensitivity. To validate these assays, initial experiments were carried out using known apoptosis and oncosis inducers. TNF-related apoptosis inducing ligand (TRAIL)–treatment of the HCT116 colorectal cancer cell line was performed as a positive control for apoptosis, and heatshock and kahalalide F treatment of HepG2 cells was performed as a positive control for oncosis.

4.2.1 Adenylate Nucleotide Ratio Assay

Bioluminescent detection and measurement of ATP, that is present in all metabolically active cells, has been undertaken since the mid-1980s (Kangas et al, 1984; Higashi et al 1985). Initially, solely ATP was measured by this method to determine cell viability, however more recently it has been reported that by measuring levels of both ADP and ATP in cells it is possible to discriminate between apoptotic and oncotic cell death (Bradbury et al, 2000). ATP levels are simply measured by bioluminescence: luciferase consumes ATP to convert luciferin to light. ADP can be converted into ATP and by making 2 successive measurements (before and after addition of ADP-CR), an indirect measurement of ADP levels and a direct measurement of ATP is provided.

The adenylate nucleotide ratio assay from Cambrex, ApoGlow™, was initially selected to measure both ADP and ATP in cells, but proved to be unreliable and results were not reproducible (see chapter 6). The CellTiter-Glo® assay (Promega) was therefore used to measure ATP levels in cells, followed by the use of ADP converting reagent (ADP-CR) from the Cambrex ApoGlow™ kit to measure ADP.

In order to confirm the finding of Bradbury et al (2000) that ADP and ATP levels could be used to discriminate between cells dying by apoptosis or oncosis, preliminary experiments were carried out. HepG2 cells were heat-shocked for 1 hour at 57°C to induce oncosis. Heat-shock results in massive cell death, and ATP depletion as demonstrated in figure 4.2. ADP levels are in fact higher than ATP levels in heat-shocked cells, resulting in a high ADP:ATP ratio as reported by Bradbury et al (2000). Control cells showed an ADP:ATP ratio of 0.25, lower than 1 as expected. In heat-shocked cells, the ADP levels were higher than ATP levels, and ADP:ATP ratio was 4.8 similar to ADP:ATP ratios reported in oncotic cells by Bradbury et al. However this is mainly due to ATP depletion subsequent to massive cell death as can be seen in figure 4.2.

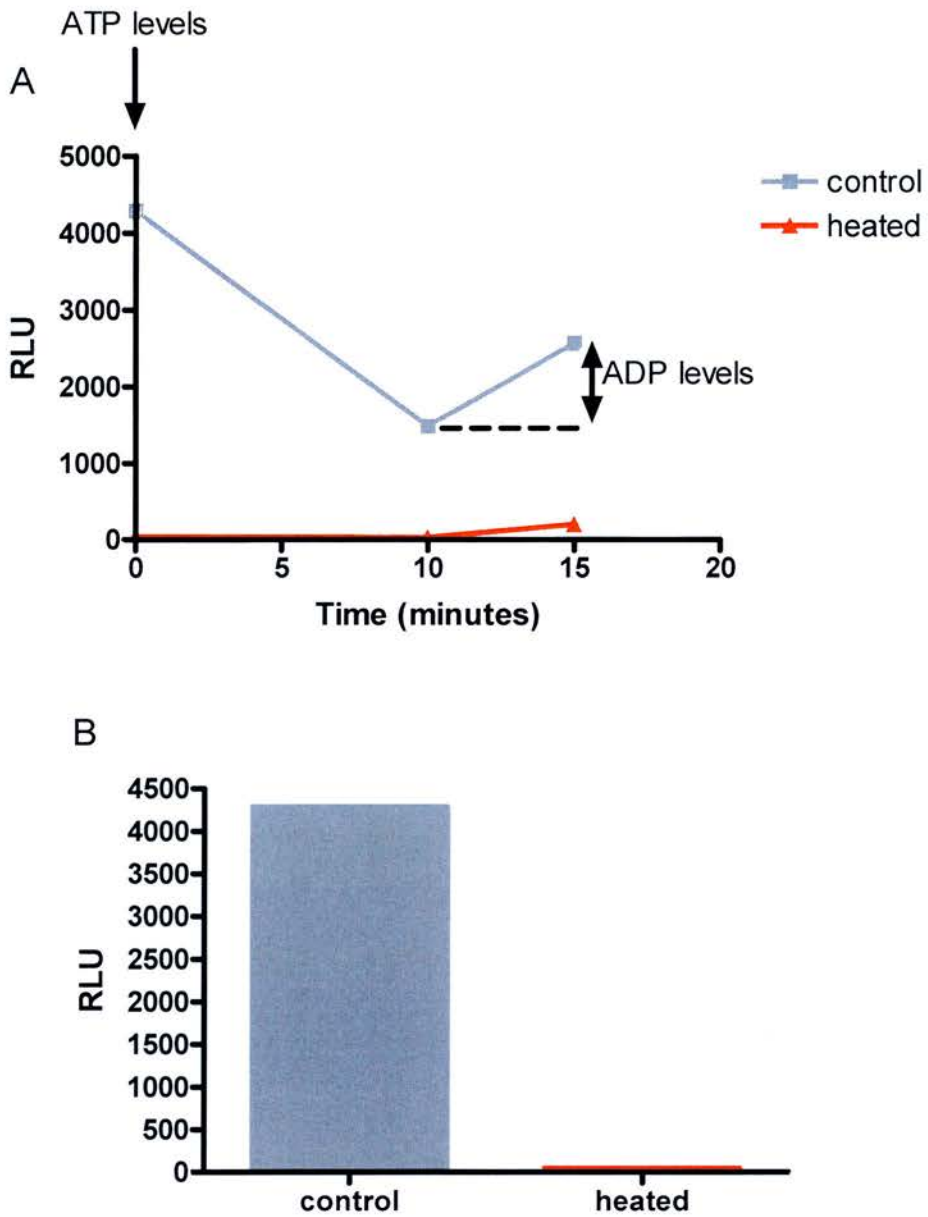


Figure 4.2: ADP and ATP levels in HepG2 cells after heat-shock

HepG2 cells were incubated at 37°C (control) or at 57°C (heated) for 1 hour, before 5000 cells were aliquoted per well and ADP and ATP assay performed. A, raw RLU data from luminometer. B, ATP levels.

To assess whether ADP:ATP ratios could discriminate oncosis from apoptosis, HepG2 cells were exposed to increasing concentrations of Kahahlide F; an anticancer drug previously shown to induce oncosis (Sewell et al, 2005). Profound ATP depletion occurred in a dose-dependent manner from 99% of control after

0.05μM of kahalalide F to 15% after 1 μM and <1% after 5 μM drug, and ADP depletion followed the same trend. The ADP:ATP ratio increases only slightly at 1 and 5 μM Kahalalide F (0.81 and 0.7 respectively), compared with 0.61 in control cells and these ratios are not of the same magnitude of changes as those reported by Bradbury et al (2000) in cells dying by oncosis.

As a low ADP:ATP ratio has been reported to correspond to apoptotic cell death (Bradbury et al, 2000), HCT116 cells were treated with TRAIL, an agent known to induce apoptosis (figure 4.4). After treatment of HCT116 cells with TRAIL for 2.5 hours, levels of ATP and ADP were normalised to SRB assay data (to account for differences in cell number) and did not change significantly with increasing TRAIL concentration. HCT116 cells did not show any change in ATP levels except at the highest concentration of TRAIL (50ng/ml) by which time 40% of the cells had died (determined via SRB assay) (figure 4.4). ADP:ATP ratios did not change compared to control (~0.66) whatever the TRAIL concentration although a slight increase (0.77) was observed after exposure to 50ng/ml of TRAIL.

This study therefore shows that, under the conditions tested, the ratio of ADP:ATP could not discriminate between apoptosis and oncosis as previously published. The Apoglow™ kit from Cambrex and the paper by Bradbury et al (2000) reported ADP:ATP ratios of 0.1 in control cells, 0.11-1 in celld dying by apoptosis and ADP:ATP ratios >15 in cells dying via an oncotic mechanism. Despite the ADP:ATP assays not being used for the studies presented in this thesis, ATP levels may inform whether or not profound ATP depletion leading to oncosis would take place.

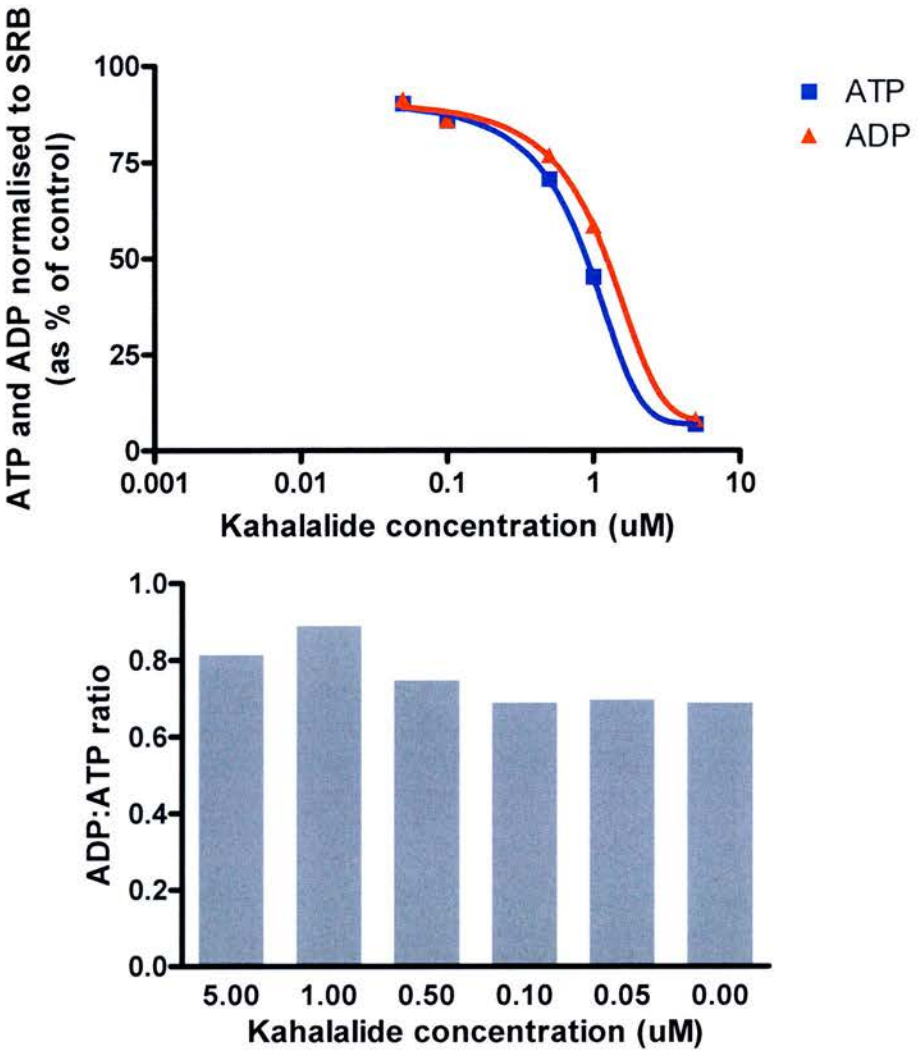


Figure 4.3: Oncosis induction by Kahalalide F in HepG2 cells

13000 HepG2 cells were plated per well, and allowed to attach overnight. Cells were then treated with a concentration range of kahalalide F for 24 hours before ADP and ATP assay.

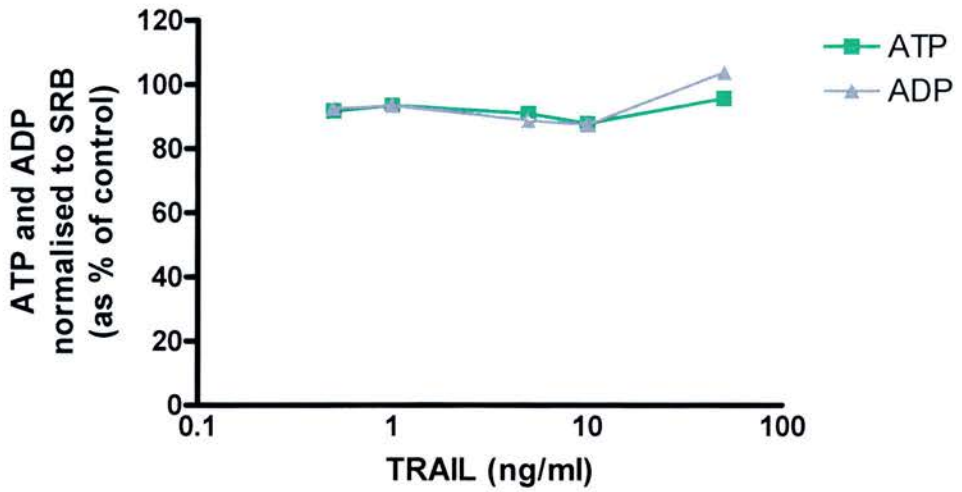


Figure 4.4: ADP and ATP levels in HCT116 cells after TRAIL exposure

25000 HCT116 cells were plated per well, and allowed to attach overnight. Cells were then treated with a concentration range of TRAIL for 2.5 hours before ADP and ATP assay.

4.2.2 Caspase-3/7 Assay

Caspase-3/7 activity was measured using the Apo-ONE® Homogeneous Caspase-3/7 assay from Promega. Induction of apoptosis has been reported in the HCT116 colon cancer cell line, following treatment with tumour necrosis factor-related apoptosis inducing ligand (TRAIL) (Cummins et al, 2004) and was used as a positive control. HepG2 cells exposed to Kahalalide F were used as a negative control.

TRAIL induced a dose-dependent and linear increase in caspase-3/7 in HCT116 cells (see figure 4.5) from 120% of control at 0.5ng/ml TRAIL to 970% at 10ng/ml consistent with reports of apoptosis induction. The caspase-3/7 assay was also tested with Kahalalide F, known to induce cell death via oncosis rather than apoptosis.

Levels of caspase-3/7 remained comparatively low even at high concentrations of Kahalalide F (300% at 5 μM)(see figure 4.6). This suggests that apoptosis if present, is limited after Kahalalide F exposure using the caspase-3/7 assay, which is consistent with published reports (Sewell et al, 2005).

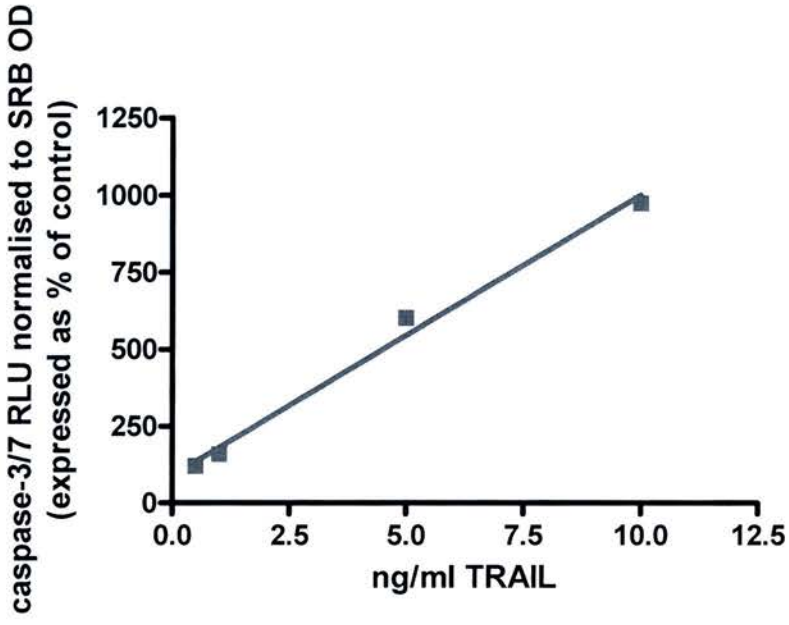


Figure 4.5: Induction of apoptosis in HCT116 cells treated with TRAIL

HCT116 cells (25000 per well) were plated and left overnight to attach. Cells were exposed to TRAIL (0.5ng/ml to 10ng/ml) for 2.5 hours before caspase-3/7 reagent was added. Measurements were made 21hours after addition of caspase-3/7 reagent. Results are presented as mean and S.D. of triplicates.

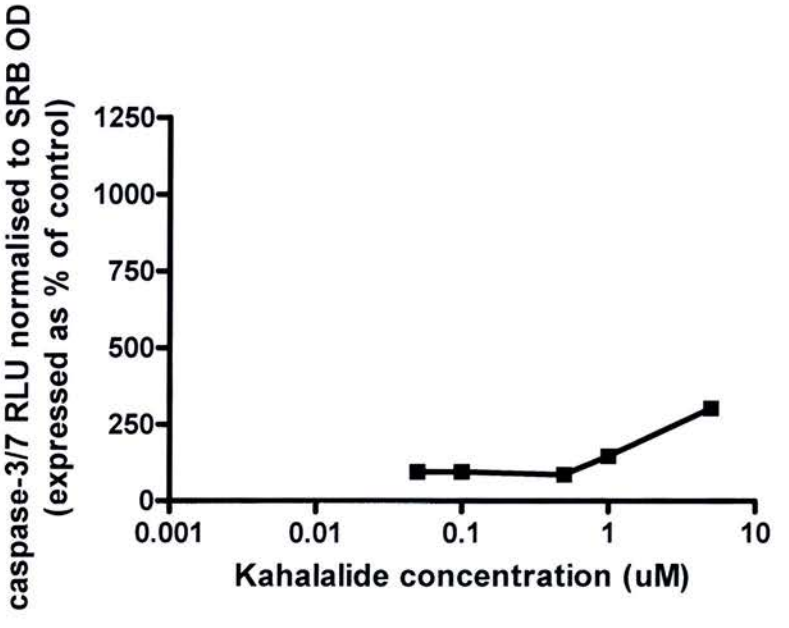


Figure 4.6: Caspase-3/7 activity in HepG2 cells exposed to kahalalide F

13000 HepG2 cells were plated per well, and allowed to attach overnight. Cells were then treated with a concentration range of kahalalide F for 24 hours before caspase-3/7 assay. Results are presented as mean and S.D. of triplicates.

4.3 ATP LEVELS IN CELLS FOLLOWING DRUG TREATMENT

ATP levels were measured in hepatoma cell lines and human hepatocytes following 24-hour drug treatment with mithramycin, oxaliplatin and 5-FU. Following drug treatment for 24 hours, a proportion of the cells treated with some of the higher drug concentrations in all cell lines and types had died, as detected by the SRB assay. In order to determine the ATP levels within the cells that were living at the end of drug treatment compared to the ATP levels in untreated control cells, the results obtained from the ATP assay were normalised to the results from the SRB assay.

4.3.1 ATP Levels in Cells Following Mithramycin Treatment

Hepatoma cell lines were treated with a concentration range of mithramycin for 24 hours before determination of ATP levels in cells (see figure 4.7). Both Huh-7D12 and PLC/PRF/5 showed an increase in intracellular ATP content at the lowest concentration of mithramycin tested (0.1μM), which decreased slightly in both cell lines with increasing mithramycin concentration. However, ATP content was still greater than in the control cells at the highest drug concentration tested (25μM) for both cell lines. Hep3B2.1-7 and Hep G2 cells maintained similar ATP levels to control cells at the lowest drug concentration tested (0.1μM), which decreased with increasing drug concentration to 67% and 93% of the ATP content respectively when compared to control cells, at the highest mithramycin concentration tested (25μM) suggesting that oncosis was not occurring.

The hepatocytes (fresh and cryopreserved) were also treated with increasing concentrations of mithramycin for 24 hours before determination of intracellular ATP levels (see figure 4.8). Donors 12 and 13 responded similarly to increasing concentrations of mithramycin, with ATP approximately 100% of control ATP levels at the lowest drug concentration investigated (0.01μM) and decreasing ATP content with increasing mithramycin concentration. At the highest mithramycin concentration tested (25μM), ATP content was at its lowest and was 71% and 87% in hepatocytes from donors 12 and 13 respectively.

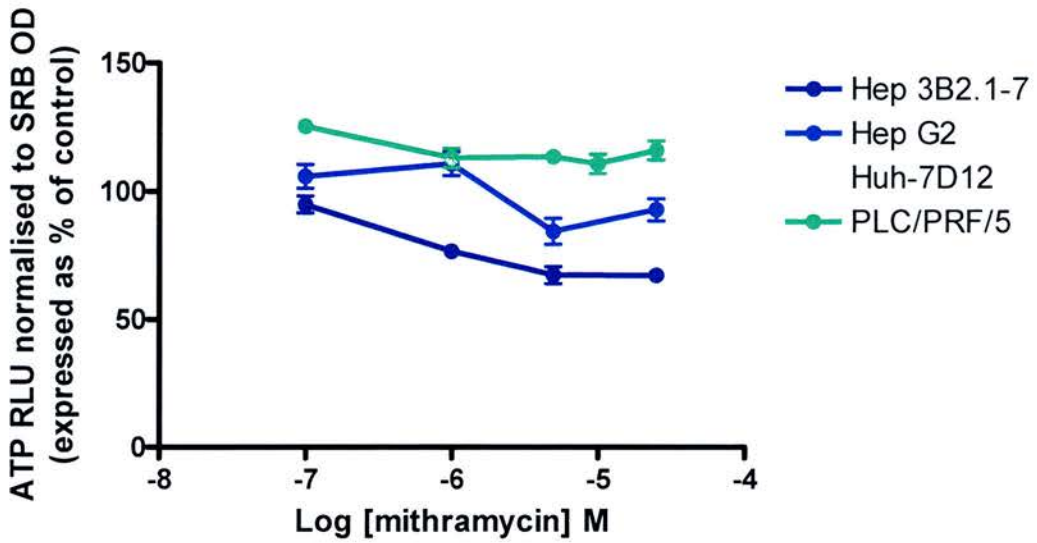


Figure 4.7: ATP levels following mithramycin treatment in hepatoma cell lines

Cell were plated (5000cells/well for Hep3B, HepG2, Huh-7D12 and 3000cells/well for PLC/PRF/5) and allowed to attach overnight before treatment with a range of mithramycin concentrations. At the end of drug treatment CellTiter-Glo reagent was added and RLU measured. Results are expressed as mean and S.E.M. of at least two independent experiments.

The cryopreserved hepatocytes from donors C2, C3, and C4 were limited in availability and therefore selected doses of each drug were used to treat these cells rather than a full concentration range of drug concentrations (see figure 4.8). At the lowest drug concentration tested (1μM), hepatocytes from donors C2 and C4 responded similarly to one another and to the fresh hepatocytes, with ATP content being approximately equal to that of untreated control cells. Donor C3 responded differently to the other hepatocytes and the ATP content was approximately 35% of that in control cells when treated with 1μM mithramycin. At the highest mithramycin concentration investigated (25μM), ATP content in cryopreserved hepatocytes varied between donors: 88%, 43% and 102% for donors C2, C3 and C4 respectively, when compared with untreated control cells.

When comparing the different models in terms of ATP content in response to mithramycin treatment, hepatocytes from donors 12, 13, C2 and C4 appear to respond similarly to increasing concentration of mithramycin as Hep3B2.1-7 and Hep G2 cell lines.

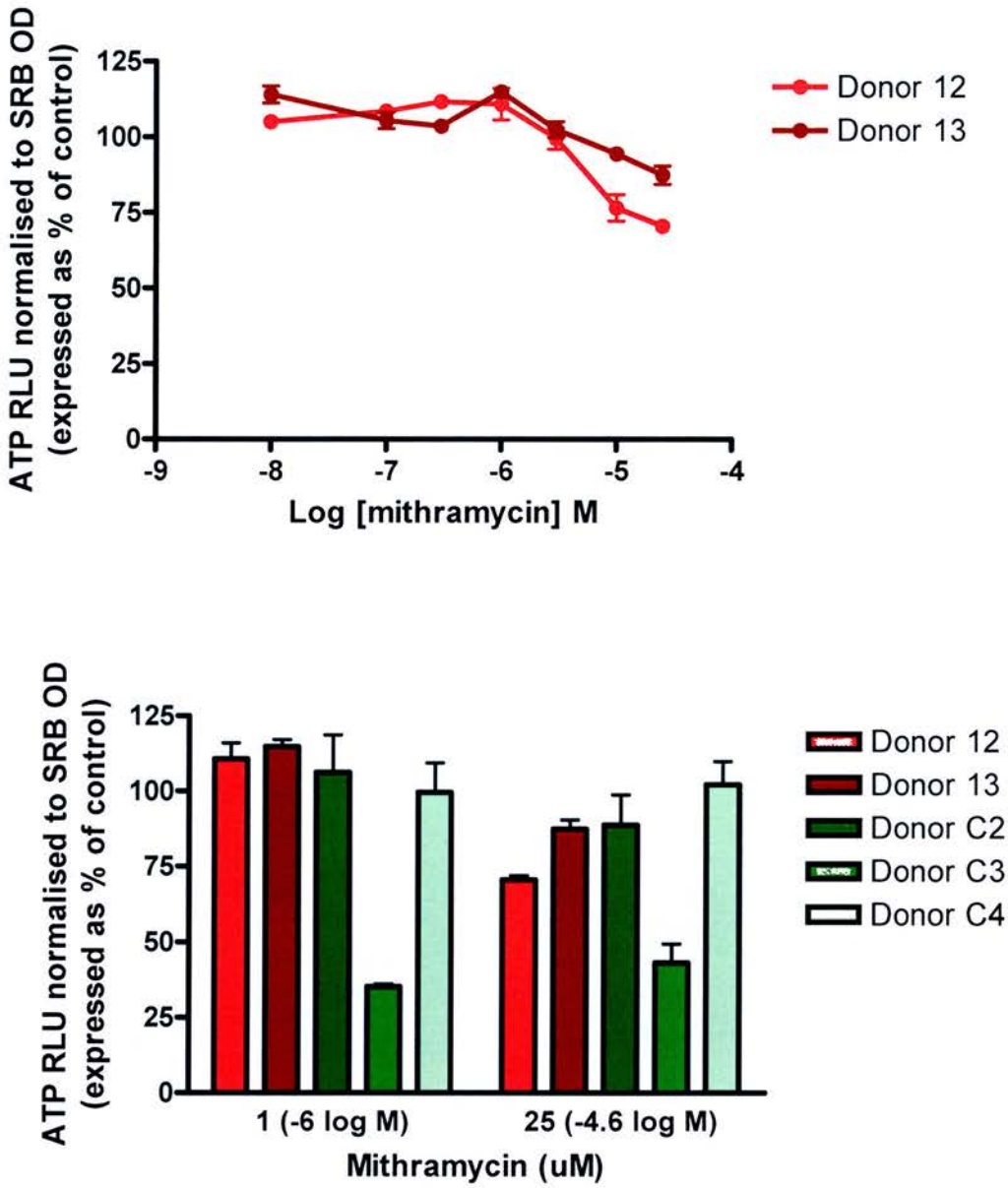


Figure 4.8: ATP levels following mithramycin treatment in hepatocytes

Cells were plated (20000 viable cells/well) from 2 fresh hepatocyte donors (12 and 13) and 3 cryopreserved hepatocyte donors (C2, C3 and C4) and allowed to attach before treatment with a range of mithramycin concentrations. At the end of drug treatment CellTiter-Glo reagent was added and RLU measured. Results are expressed as mean and S.E.M. of triplicates.

4.3.2 ATP Levels in Cells Following Oxaliplatin Treatment

Hepatoma cell lines were treated with a concentration range of oxaliplatin for 24 hours before determination of ATP levels in cells (see figure 4.9). Huh-7D12,

PLC/PRF/and Hep3B2.1-7 responded similarly to one another in response to increasing oxaliplatin concentrations. From 5μM to 50μM little change in ATP content was seen with increasing oxaliplatin concentration, however increasing oxaliplatin concentration to 250μM resulted in loss of cellular ATP content to approximately 50% of control ATP content for Hep3B2.1-7 and Huh-7D12 and to approximately 60% of control ATP content in PLC/PRF/5 cells. For comparison, the mean IC_{50} in the hepatoma cell lines at 96-hours as determined in chapter 3 was 1.17μM. HepG2 cells demonstrated a slight increase in ATP content when compared to untreated control cells at all oxaliplatin concentrations investigated.

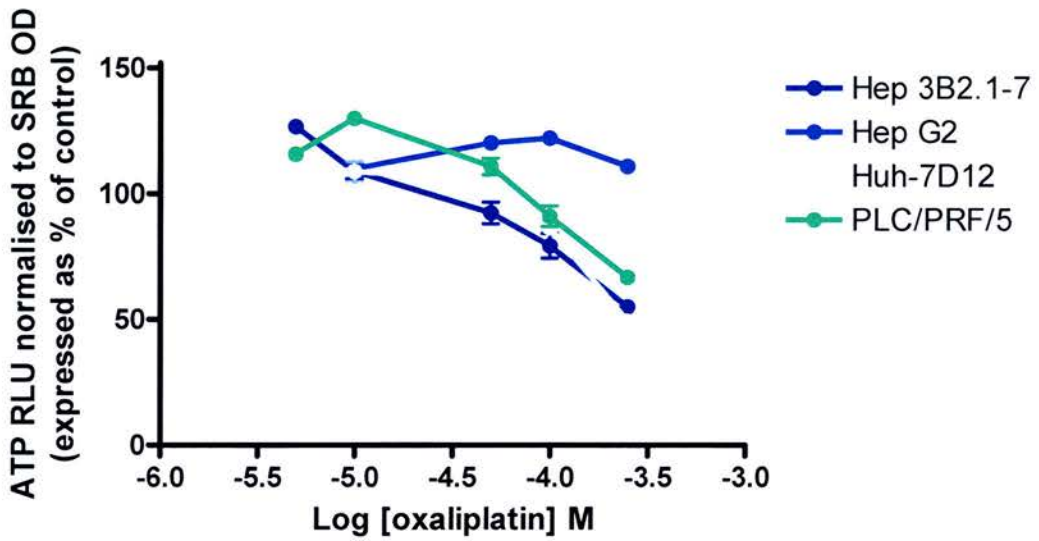


Figure 4.9: ATP levels following oxaliplatin treatment in hepatoma cell lines

Cell were plated (5000cells/well for Hep3B, HepG2, Huh-7D12 and 3000cells/well for PLC/PRF/5) and allowed to attach before treatment with a range of oxaliplatin concentrations. At the end of drug treatment CellTiter-Glo reagent was added and RLU measured. Results are expressed as mean and S.E.M. of at least two independent experiments.

Fresh hepatocytes from donors 12 and 13 showed a similar response following treatment with increasing concentrations of oxaliplatin as Huh-7D12, PLC/PRF/and Hep3B2.1-7 cell lines (figure 4.10). Little difference in ATP content when compared to untreated control cells was seen until a concentration of 50μM oxaliplatin was reached, after which increasing oxaliplatin concentration resulted in decreased cellular ATP content. The cryopreserved hepatocytes from donors C2, C3 and C4 varied widely to one another in response to treatment with oxaliplatin. Donor C3

was the most sensitive to oxaliplatin with over 80% ATP loss compared to control after treatment with 250μM oxaliplatin.

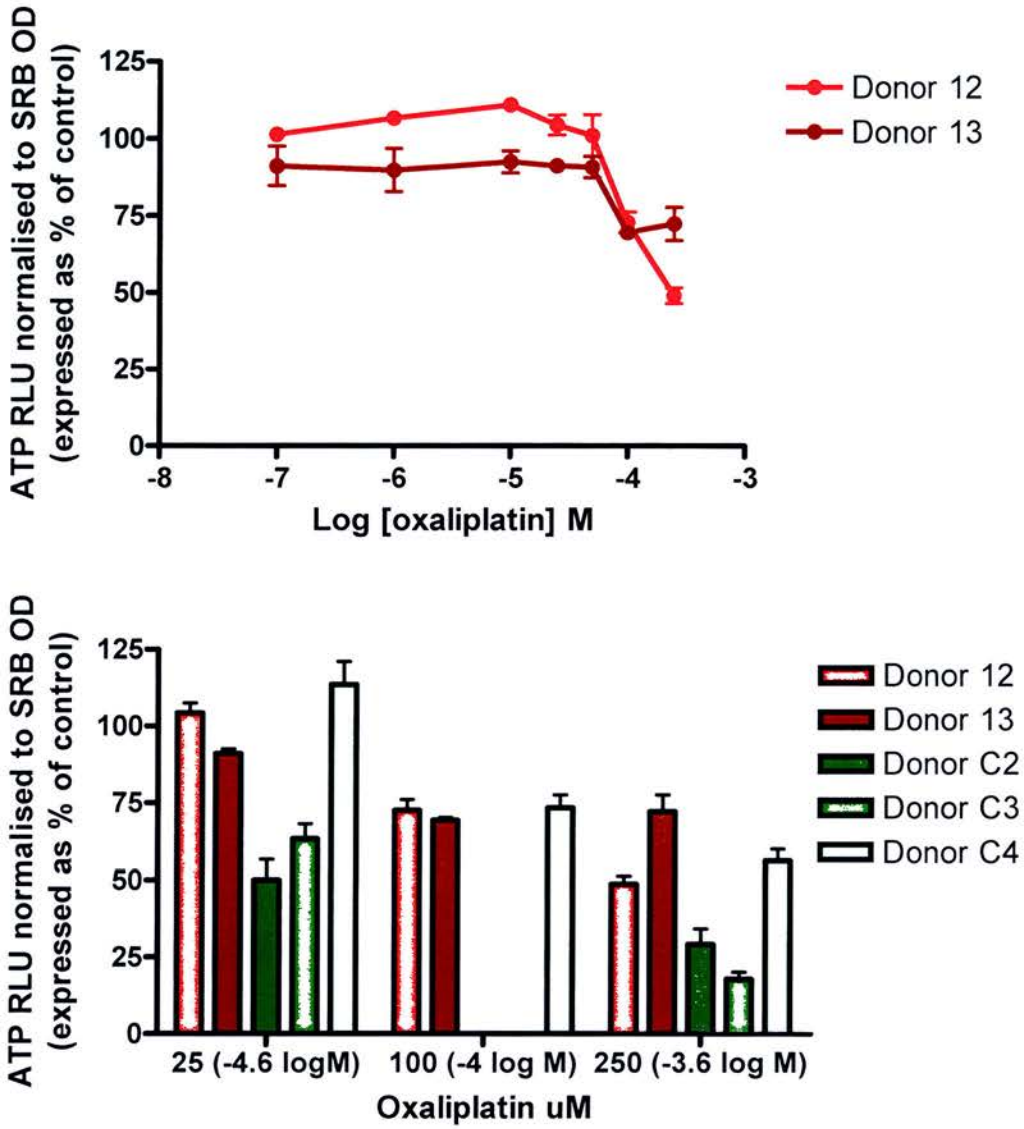


Figure 4.10: ATP levels following oxaliplatin treatment in hepatocytes

Cells were plated (20000 viable cells/well) from 2 fresh hepatocyte donors and 3 cryopreserved hepatocyte donors and allowed to attach before treatment with a range of oxaliplatin concentrations. At the end of drug treatment CellTiter-Glo reagent was added and RLU measured. Results are expressed as mean and S.E.M. of triplicates.

4.3.3 ATP Levels in Cells Following 5-FU Treatment

Hepatoma cell lines were treated with a concentration range of 5-FU for 24 hours before determination of ATP levels in cells. Hep3B2.1-7, PLC/PRF/5 and HepG2 responded similarly to one another in response to increasing 5-FU concentrations

(figure 4.11). From 10μM to 100μM little change in ATP content was seen with increasing 5-FU concentration, however increasing 5-FU concentration to 10000μM resulted in loss of cellular ATP content to approximately 50% of control ATP content in all three cell lines. Huh-7D12 cells showed the same trend of ATP loss with increasing 5-FU concentrations; however ATP loss reached a maximum of 82% of control at the highest 5-FU concentration investigated (10000μM) in Huh-7D12 cells.

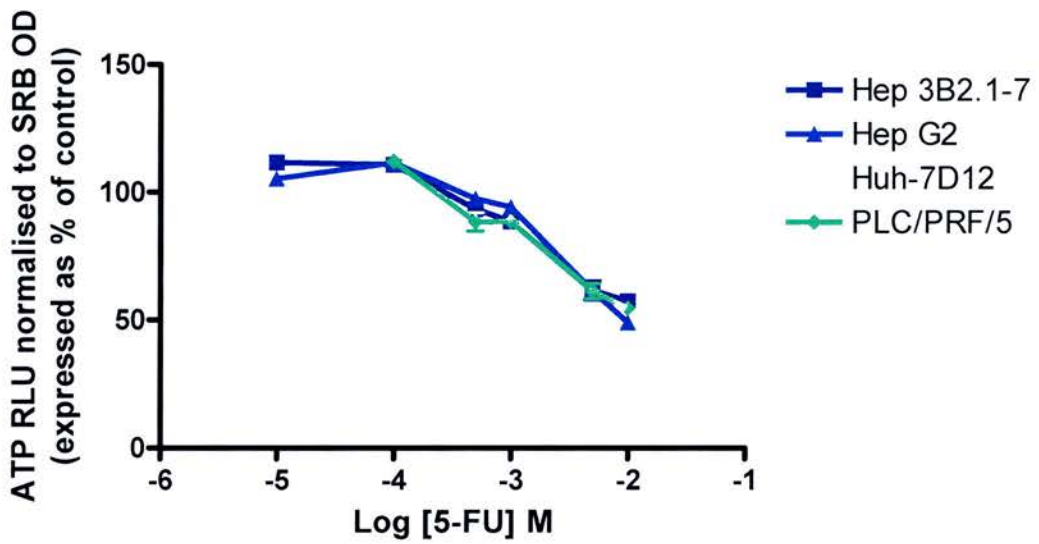


Figure 4.11: ATP levels following 5-FU treatment in hepatoma cell lines

Cells were plated (5000cells/well for Hep3B, HepG2, Huh-7D12 and 3000cells/well for PLC/PRF/5) and allowed to attach before treatment with a range of 5-FU concentrations for 24 hours. At the end of drug treatment CellTiter-Glo reagent was added and RLU measured. Results are expressed as mean and S.E.M. of at least two independent experiments.

Fresh hepatocytes from donors 12 and 13 responded differently to increasing 5-FU concentrations. Hepatocytes from donor 12 showed an increase in cellular ATP content over the 5-FU concentration range, 1μM to 10000μM. Hepatocytes from donor 13 showed a decrease in cellular ATP content with increasing 5-FU concentration and ATP content was 60% of that in control cells at the highest drug concentration investigated (10000μM). The cryopreserved hepatocytes from donors C2, C3 and C4 varied in response to treatment with 5-FU, but intracellular ATP content decreased in hepatocytes from all 3 donors with increasing 5-FU

concentration. Greatest ATP loss compared to control was observed in cryopreserved hepatocytes from donor C3 treated with 10mM 5-FU (53%) followed by donor C4 (65%) and C2 (77%).

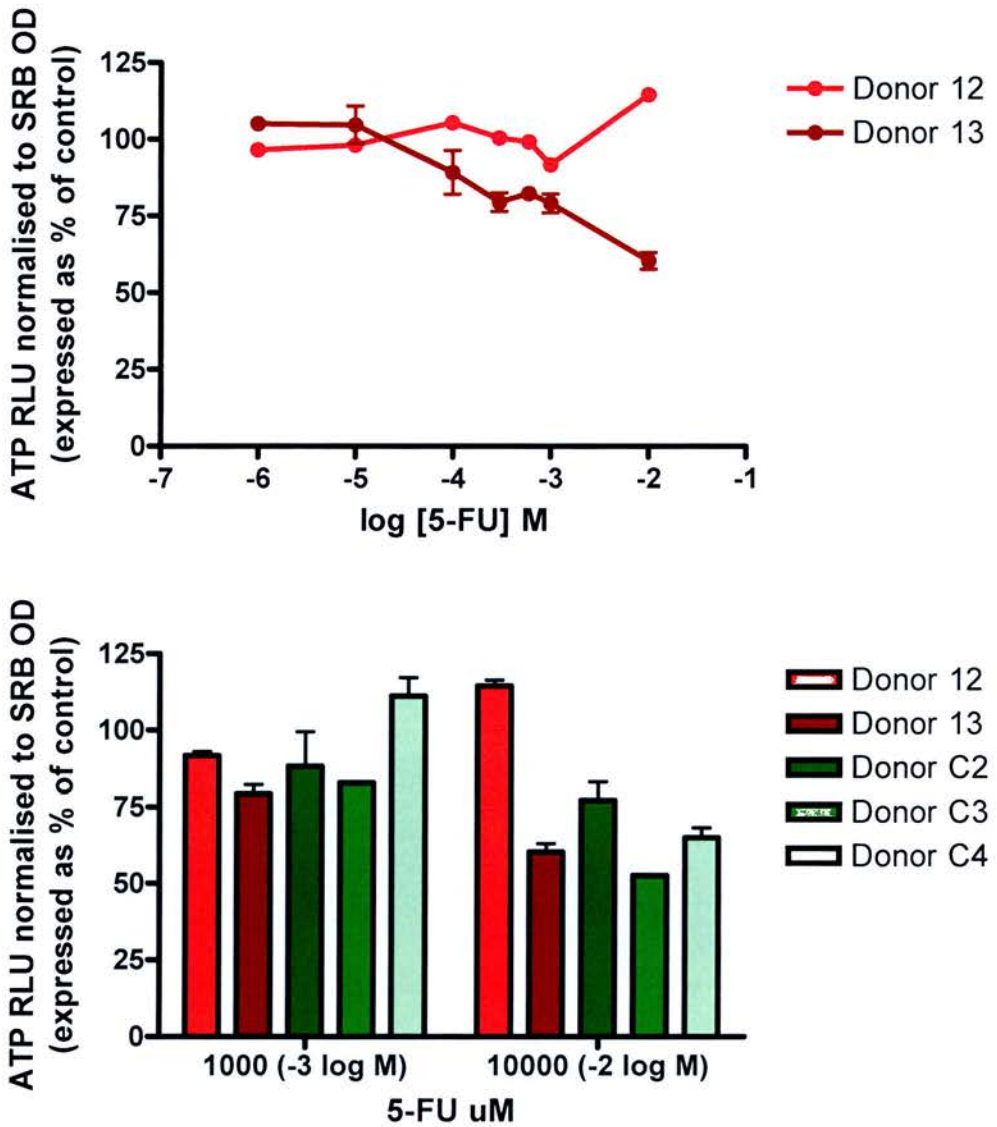


Figure 4.12: ATP levels following 5-FU treatment in hepatocytes

Cells were plated (20000 viable cells/well) from 2 fresh hepatocyte donors and 3 cryopreserved hepatocyte donors and allowed to attach before treatment with a range of 5-FU concentrations. At the end of drug treatment CellTiter-Glo reagent was added and RLU measured. Results are expressed as mean and S.E.M. of triplicates.

When considering all three of the drugs tested, ATP depletion was limited in the hepatoma cell lines : Hep3B2.1-7 was the only cell line showing significant decrease of ATP for all drugs tested: 57% at 10mM 5-FU, 55% at 250μM oxaliplatin and 67%

at 25μM mithramycin. These results suggest that oncosis, inducing profound ATP depletion, does not occur in hepatoma cell lines after drug exposure unless concentrations far beyond the IC_{50}s determined in chapter 3 are applied. When considering ATP levels for all three drugs in fresh hepatocytes from both donors, for mithramycin and oxaliplatin the response in terms of ATP levels were similar, whereas in response to 5-FU, ATP loss was observed in hepatocytes from donor 13 and not donor 12. As for the hepatoma cell lines, substantial ATP loss was only observed at concentrations far higher than the IC_{50}s determined in chapter 3.

4.4 CASPASE-3/7 ACTIVATION IN CELLS FOLLOWING DRUG TREATMENT

Profound ATP depletion does not occur after exposure of the different cell models to mithramycin, oxaliplatin or 5-FU, suggesting that apoptosis rather than oncosis is induced by these drugs at cytotoxic concentrations. In order to confirm this hypothesis, Caspase-3/7 levels were measured in the hepatocyte models following 24-hour drug treatment with mithramycin, oxaliplatin and 5-FU.

4.4.1 Caspase-3/7 Activation Following Mithramycin Treatment

Hepatoma cell lines were treated with a concentration range of mithramycin for 24 hours before determination of caspase-3/7 levels in cells (see figure 4.13). PLC/PRF/5 and Hep3B2.1-7 responded similarly to increasing mithramycin concentrations, with caspase-3/7 activity increasing with increasing mithramycin concentration. The activation peaked at 1μM mithramycin in Hep3B2.1-7 cells, and at 10μM mithramycin in PLC/PRF/5 to an approximately 6-fold increase compared to control in both cases. In these cell lines, caspase activation was lower at the highest concentrations at 4 and 5.2-fold in Hep3B2.1-7 and PLC/PRF/5 respectively. The Hep G2 and Huh-7D12 cell lines responded very similarly to one another in response to increasing mithramycin concentrations with a dose-dependent but limited increase in caspase-3-7 activity to approximately 2-fold when compared with control cells at the maximum mithramycin concentration tested (25μM).

In hepatocytes from donors 12 and 13 the response to increasing mithramycin concentration in terms of caspase-3/7 activation varied greatly (figure 4.14). Hepatocytes from donor 12 had a much larger increase in caspase-3/7 activity in response to mithramycin than hepatocytes from donor 13, and in hepatocytes from donor 12 peak caspase-3/7 activity was observed at 10μM mithramycin (15-fold). The cryopreserved hepatocytes from donors C2, C3 and C4 varied in response to treatment with mithramycin, and caspase-3/7 activation was more similar to that in hepatocytes from donor 13 than from donor 12 (figure 4.14).

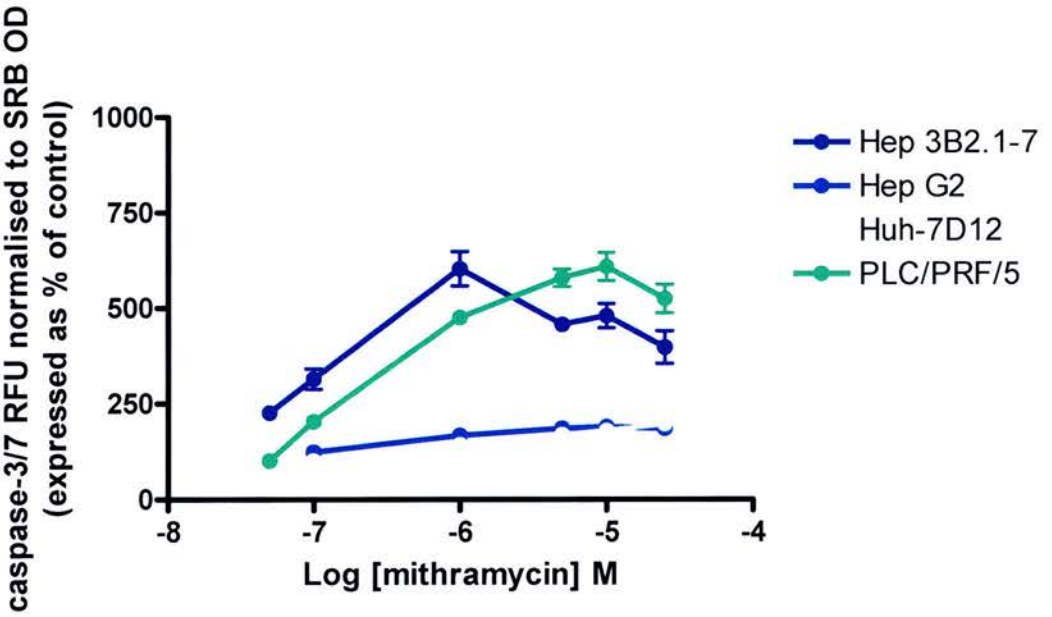


Figure 4.13: Caspase-3/7 following mithramycin treatment in hepatoma cell lines

Cells were plated (5000cells/well for Hep3B, HepG2, Huh-7D12 and 3000cells/well for PLC/PRF/5) and allowed to attach overnight before treatment with a range of mithramycin concentrations. At the end of drug treatment caspase-3/7 reagent was added and RFU measured after 21 hours. Results are expressed as mean and S.E.M. of at least two independent experiments.

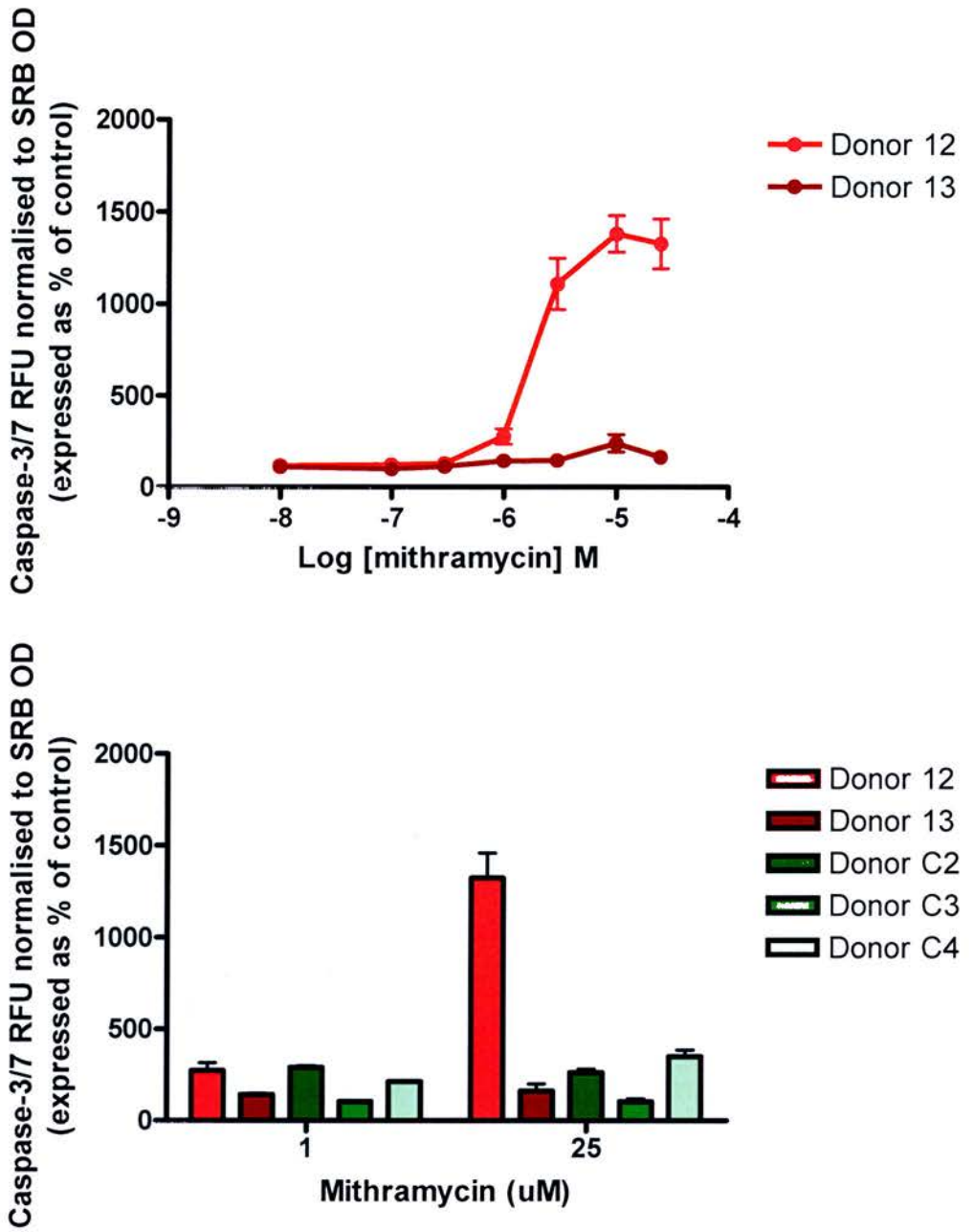


Figure 4.14: Caspase-3/7 levels following mithramycin treatment in hepatocytes

Cells were plated (20000 viable cells/well) from 2 fresh hepatocyte donors and 3 cryopreserved hepatocyte donors and allowed to attach overnight before treatment with a range of mithramycin concentrations. At the end of drug treatment caspase-3/7 reagent was added and RFU measured after 21 hours. Results are expressed as mean and S.E.M. of triplicates.

4.4.2 Caspase-3/7 Activation Following Oxaliplatin Treatment

Hepatoma cell lines were treated with a concentration range of oxaliplatin for 24 hours before determination of caspase-3/7 levels in cells. All four of the hepatoma cell lines investigated demonstrated a dose-dependent increase in caspase-3/7 activation beginning at the oxaliplatin concentration of 5μM (see figure 4.15). HepG2 cells showed caspase activation (5-fold) at the lowest oxaliplatin concentration (10μM) compared to the other cells lines. However, concentrations of oxaliplatin greater than 10μM did not result in further caspase activation in HepG2 cells. In the other hepatoma cell lines, maximum caspase activation in response to oxaliplatin was observed at 50μM, 100μM and 250 μM in Huh-7D12 (6.5-fold), Hep3B2.1-7 (8-fold) and PLC/PRF/5 (5-fold) respectively.

Similar to mithramycin response, in hepatocytes from donors 12 and 13 the response to increasing oxaliplatin concentration in terms of caspase-3/7 activation varied greatly (figure 4.16). Hepatocytes from donor 12 had a much larger increase in caspase-3/7 activity in response to oxaliplatin than hepatocytes from donor 13, and maximum activation was observed in donor 12 at 100 μM oxaliplatin (14-fold). The cryopreserved hepatocytes from donors C2, C3 and C4 varied in response to treatment with oxaliplatin, and caspase-3/7 activation was more similar to that in hepatocytes from donor 13 than from donor 12 (figure 4.16).

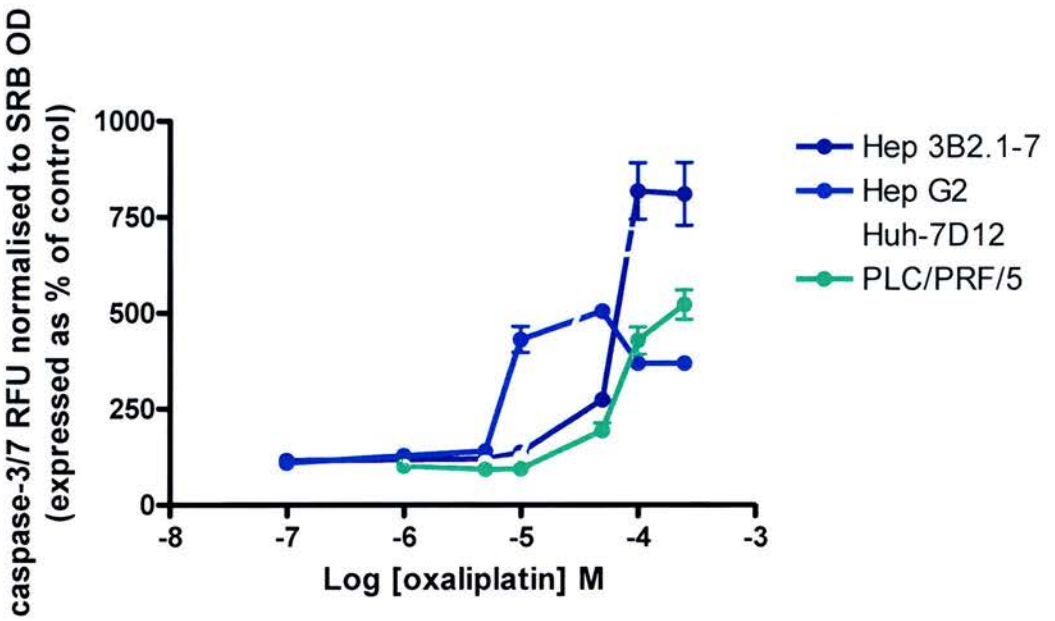


Figure 4.15: Caspase-3/7 levels following oxaliplatin treatment in hepatoma cell lines

Cells were plated (5000cells/well for Hep3B, HepG2, Huh-7D12 and 3000cells/well for PLC/PRF/5) and allowed to attach overnight before treatment with a range of oxaliplatin concentrations. At the end of drug treatment caspase-3/7 reagent was added and RFU measured after 21 hours. Results are expressed as mean and S.E.M. of at least two independent experiments.

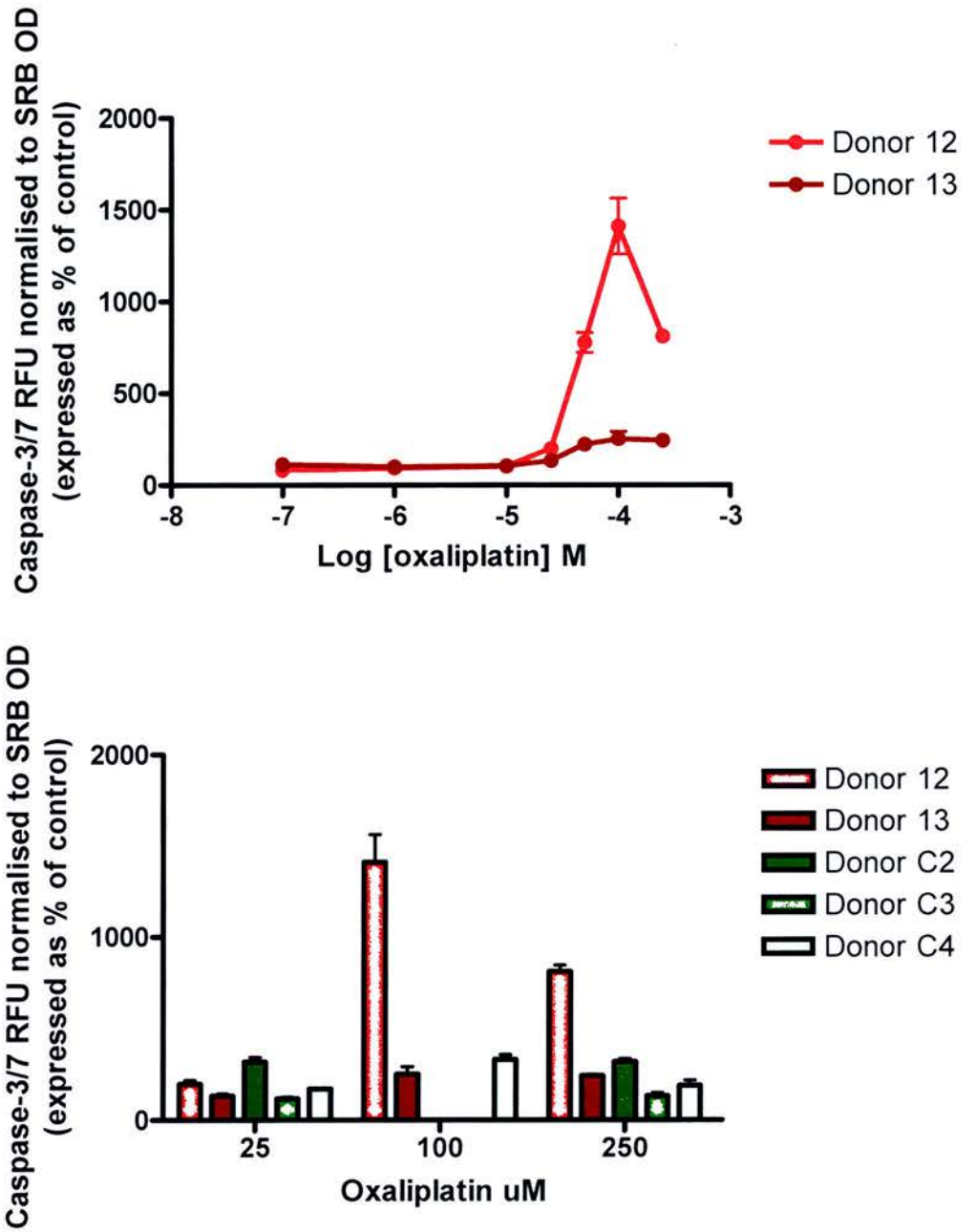


Figure 4.16: Caspase-3/7 levels following oxaliplatin treatment in hepatocytes

Cells were plated (20000 viable cells/well) from 2 fresh hepatocyte donors and 3 cryopreserved hepatocyte donors and allowed to attach overnight before treatment with a range of oxaliplatin concentrations. At the end of drug treatment caspase-3/7 reagent was added and RFU measured after 21 hours. Results are expressed as mean and S.E.M. of triplicates.

4.4.3 Caspase-3/7 Activation Following 5-FU Treatment

Hepatoma cell lines were treated with a concentration range of 5-FU for 24 hours before determination of caspase-3/7 levels in cells, and the results are presented in

figure 4.17. Huh-7D12 showed no change in caspase-3/7 activity with increasing 5-FU concentration. PLC/PRF/5 and Hep3B2.1-7 demonstrated a small dose-dependent increase in caspase-3/7 activity with increasing 5-FU concentration. Caspase-3/7 activity increased in Hep G2 cells with 5-FU concentration increasing from 1000μM to 10000 5-FUμM (to 3.5-fold).

Similar to the two drugs tested previously, in hepatocytes from donors 12 and 13 the response to increasing 5-FU concentration in terms of caspase-3/7 activation varied greatly. Hepatocytes from donor 12 had a much larger increase in caspase-3/7 activity in response to 5-FU than hepatocytes from donor 13 (figure 4.18), and maximum caspase activation was observe in hepatocytes from donor 12 at 5-FU concentration of 10000μM. The cryopreserved hepatocytes from donors C2, C3 and C4 varied in response to treatment with 5-FU (figure 4.18). The cryopreserved hepatocytes from donors C2, C3 and C4 varied in response to treatment with mithramycin, oxaliplatin and 5-FU and caspase-3/7 activation was more similar to that in fresh hepatocytes from donor 13 than donor 12.

In the fresh hepatocytes, all three drugs tested induced caspase-3/7 activation in hepatocytes from donor number 12, and very limited caspase activation in hepatocytes from donor number 13. The difference between the 2 samples may be partially explained by the normalisation of caspase-3/7 data to the SRB optical density data. In hepatocytes from donor number 12, a dose-dependent increase in cell death was observed with increasing drug concentration whereas in hepatocytes from donor number 13 very little cell death was observed immediately following 24-hour drug exposure. The difference in normalised caspase-3/7 levels is therefore greater in the data from donor 12 than 13. However, decrease in SRB OD confirms cell death which is reflected by the increase in caspase-3/7 in donor 12.

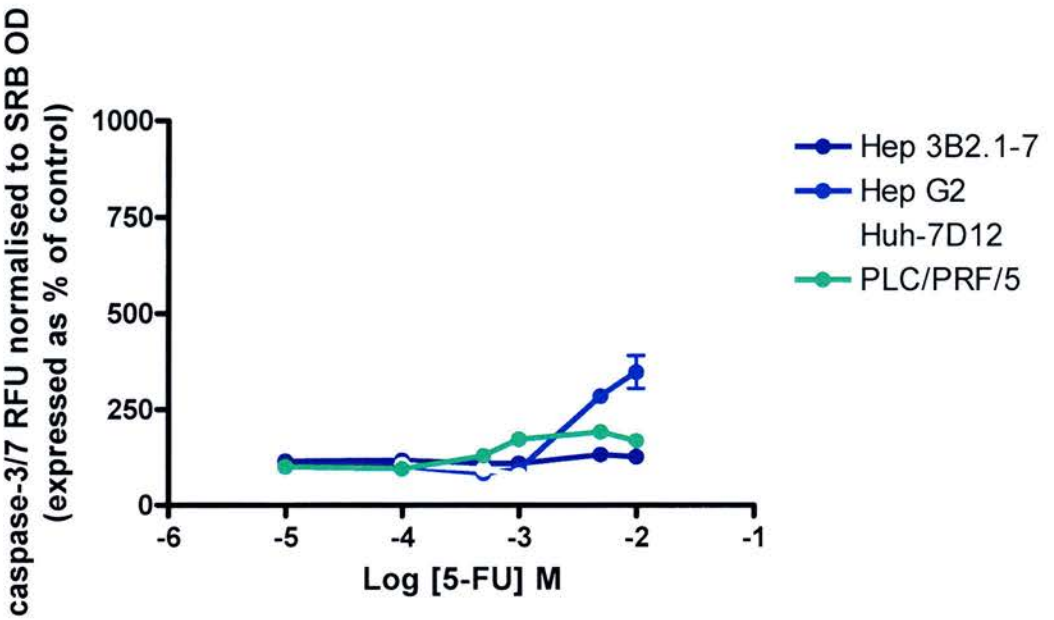


Figure 4.17: Caspase-3/7 levels following 5-FU treatment of hepatoma cell lines

Cells were plated (5000cells/well for Hep3B, HepG2, Huh-7D12 and 3000cells/well for PLC/PRF/5) and allowed to attach overnight before treatment with a range of 5-FU concentrations. At the end of drug treatment caspase-3/7 reagent was added and RFU measured after 21 hours. Results are expressed as mean and S.E.M. of at least two independent experiments.

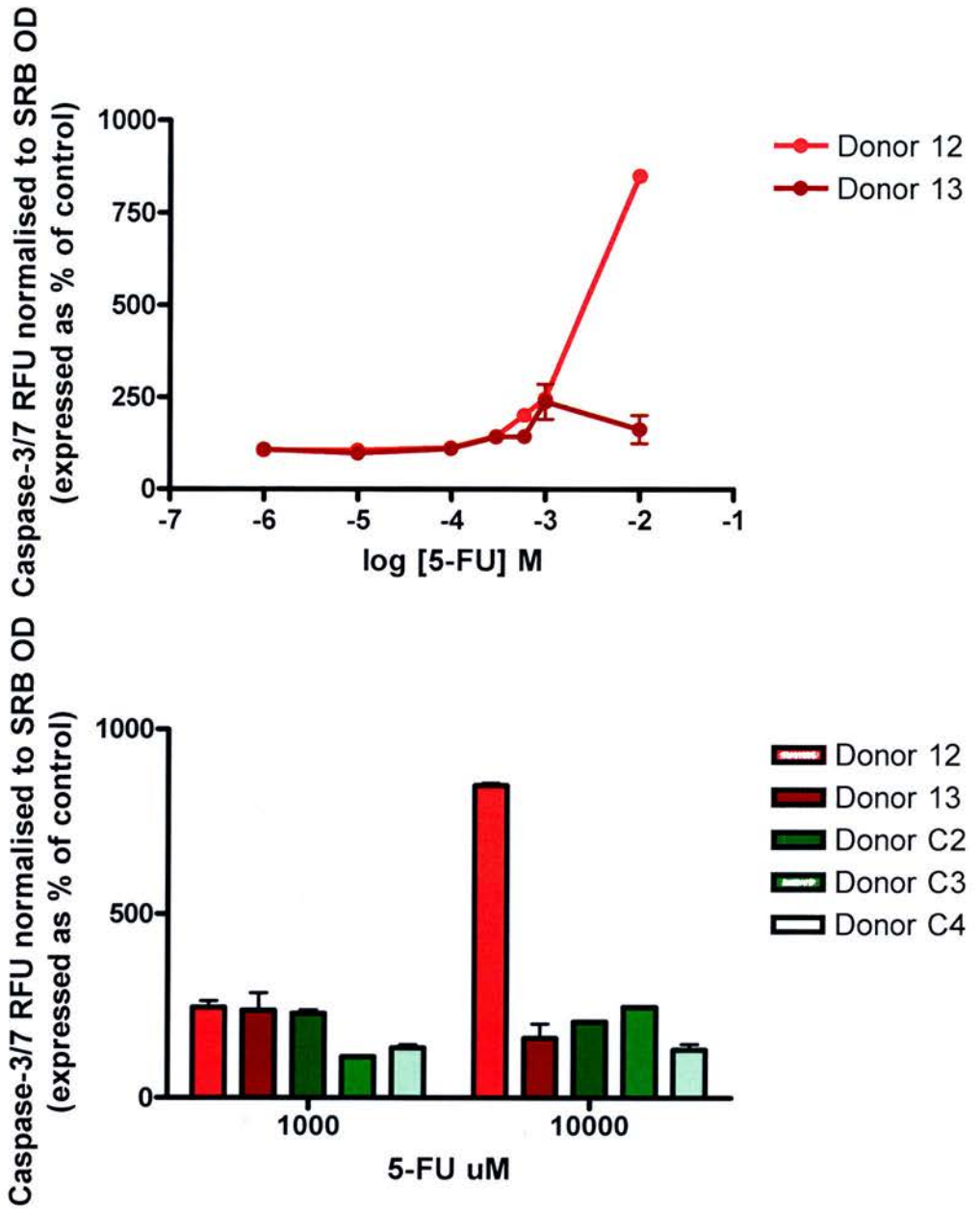


Figure 4.18: Caspase-3/7 levels following 5-FU treatment of hepatocytes

Cells were plated (20000 viable cells/well) from 2 fresh hepatocyte donors and 3 cryopreserved hepatocyte donors and allowed to attach overnight before treatment with a range of 5-FU concentrations. At the end of drug treatment caspase-3/7 reagent was added and RFU measured after 21 hours. Results are expressed as mean and S.E.M. of triplicates.

4.5 COMPARISON OF EARLY (24-HOUR) AND LATER (96-HOUR) DATA

The MTT and SRB assays provide a measure of cell number either by detecting the metabolic capacity of cells (MTT), or the amount of cellular protein (SRB).

Individually, these assays allow the detection of changes in cell number in response to drug treatment *in vitro*. Whilst useful on their own, it was also of interest to detect the earlier response of cells to treatment with anti-cancer agents. It was hypothesised that by measuring the levels of a nucleotide present in cells (ATP), immediately following drug treatment it may be possible to detect the earlier response of cells to drug-treatment. The ATP assay has been used to detect changes in drug-treated cells at the end of 24-hour drug treatment. The data from the ATP assay will be compared with the data from MTT and SRB assays at 96 hours after the start of drug treatment (in chapter 3) to determine whether the ATP assay can provide an earlier indication and the same pattern of cell death as the SRB and MTT assays at a later timepoint. For comparison with the cytotoxicity detected after 96 hours with the SRB and MTT assays, the ATP assay carried out immediately following 24-hour drug treatment will be examined.

To determine whether ATP levels (normalised to SRB) in drug-treated cells detected immediately following 24-hour drug treatment may be used as a method for earlier detection of cellular response to drug treatment (than SRB or MTT assay at 96 hours), results from ATP and SRB assays immediately following drug treatment and SRB and MTT assays at the end of drug treatment are plotted in figures 4.19 and 4.20, 4.21 and 4.22 for fresh hepatocytes and hepatoma cell lines respectively.

4.5.1 Mithramycin

In Hep3B2.1-7 cells intracellular ATP levels decrease with increasing drug concentration and in HepG2, Huh-7D12 and PLC/PRF/5 cells intracellular ATP levels do not change with increasing drug concentration. Caspase-3/7 activation increases minorly in HepG2 and Huh-7D12 cells and increases more in PLC/PRF/5 and Hep3B2.1-7 cells with increasing drug concentration. At 96 hours the results obtained using the SRB assay and MTT assays are similar in HepG2 cells and in Hep3B2.1-7, Huh-7D12 and PLC/PRF/5, at 96 hours the SRB assay is more sensitive than the MTT assay.

In hepatocytes from donor 12 and 13, intracellular ATP decreases minorly with increasing drug concentration. Caspase-3/7 activation increases with increasing drug concentration in hepatocytes from donor 12 and to a much lesser extent in hepatocytes from donor 13. At 96 hours the MTT assay is more sensitive than the SRB assay in donor 12 and the opposite is true for donor 13.

4.5.2 Oxaliplatin

In Hep3B2.1-7, Huh7D12 and PLC/PRF/5 cells intracellular ATP levels decreased with increasing drug concentration. In the same three cell lines, caspase-3/7 activation increases with increasing drug concentration, and at 96 hours the SRB assay is more sensitive than the MTT assay. In Hep G2 cells, intracellular ATP levels do not change with increasing drug concentration. Caspase-3/7 activation increases with increasing drug concentration, but to a lesser extent than in the other hepatoma cell lines. At 96 hours the SRB assay is more sensitive than the MTT assay.

In hepatocytes from donor 12, intracellular ATP decreases with increasing drug concentration, and to a lesser extent in hepatocytes from donor 13. Caspase-3/7 activation increases with increasing drug concentration in hepatocytes from donor 12 and there is no caspase-3/7 activation seen with increasing drug concentration in hepatocytes from donor 13. At 96 hours the results obtained using the SRB assay and MTT assays are similar in donor 12, and in donor 13 the SRB assay is more sensitive than the MTT assay.

4.5.3 5-FU

In Hep3B2.1-7, HepG2 and PLC/PRF/5 cells intracellular ATP levels decreased with increasing drug concentration, and the same is true in Huh-7D12 cells except intracellular ATP levels only decreased minorly. There is no caspase-3/7 activation seen with increasing drug concentration in Hep3B2.1-7 and Huh-7D12 cells, a small increase is seen in PLC/PRF/5 cells, and the greatest caspase activation in response to 5-FU is seen in HepG2 cells. At 96 hours the SRB assay is more sensitive than the MTT assay with all four hepatoma cell lines tested.

In hepatocytes from donor 12, intracellular ATP does not change with increasing drug concentration. Caspase-3/7 activation increases with increasing drug concentration. At 96 hours the MTT assay is more sensitive than the SRB assay.

In hepatocytes from donor 13, intracellular ATP decreased with increasing drug concentration. Caspase-3/7 activation increases minorly with increasing drug concentration. At 96 hours the SRB assay is more sensitive than the MTT assay.

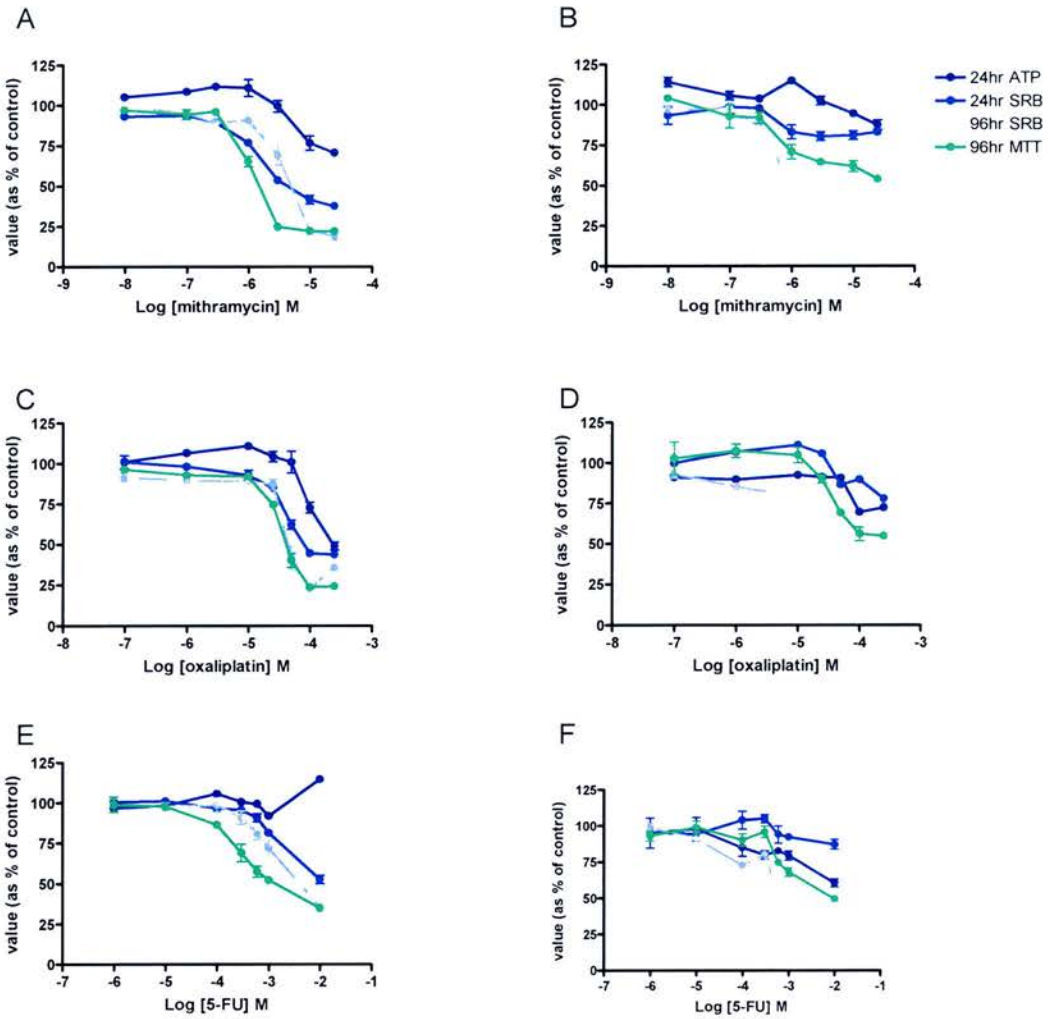


Figure 4.19: Comparison of ATP, SRB and MTT assay in fresh hepatocytes treated with mithramycin, oxaliplatin or 5-FU

Fresh hepatocytes from donors 12 (A, C, E) and 13 (B, D, F) were treated with a concentration range of mithramycin (A, B), oxaliplatin (C, D) or 5-FU (E,F) for 24 hours, followed by immediate ATP and SRB assays, and SRB and MTT assays 96 hours after the start of drug treatment. Results are expressed as mean and S.E.M. of triplicates.

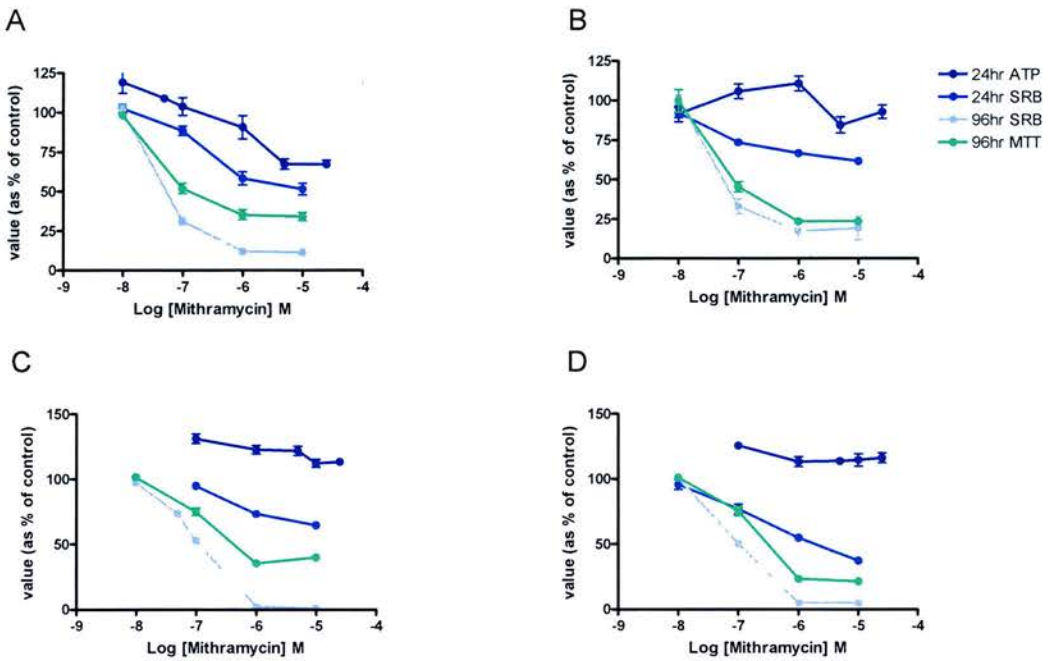


Figure 4.20: Comparison of ATP, SRB and MTT assay in hepatoma cell lines treated with mithramycin

Hep3B2.1-7 (A), Hep G2 (B), Huh-7D12 (C) and PLC/PRF/5 (D) cell lines were treated with a concentration range of mithramycin for 24 hours, followed by immediate ATP and SRB assays, and SRB and MTT assays 96 hours after the start of drug treatment. Results are expressed as mean and S.E.M. of at least two independent experiments.

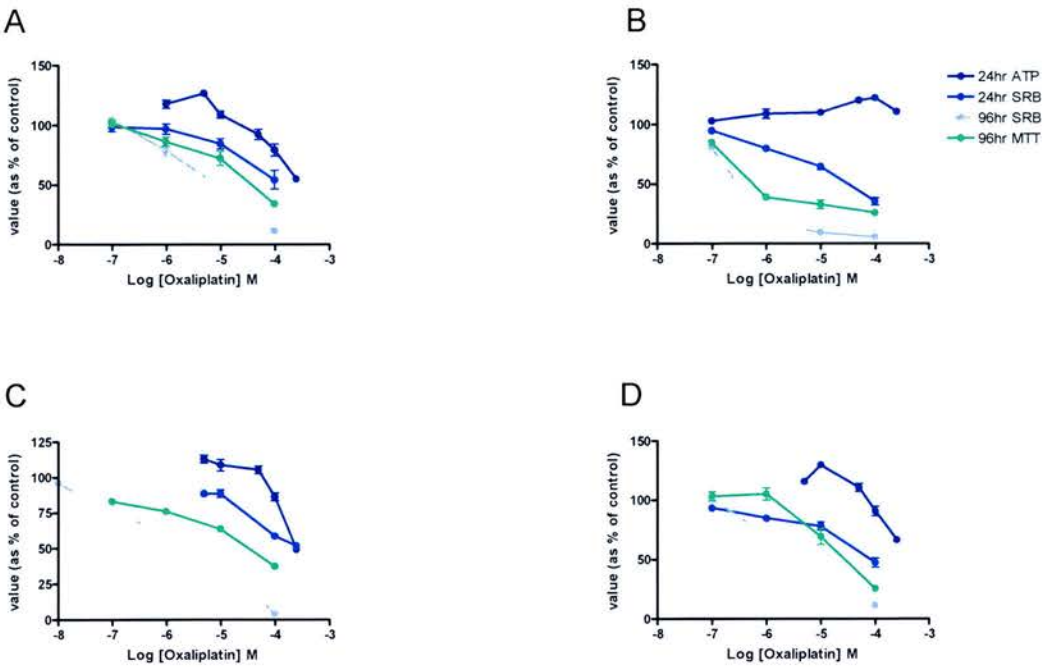


Figure 4.21: Comparison of ATP, SRB and MTT assay in hepatoma cell lines treated with oxaliplatin

Hep3B2.1-7 (A), Hep G2 (B), Huh-7D12 (C) and PLC/PRF/5 (D) cell lines were treated with a concentration range of oxaliplatin for 24 hours, followed by immediate ATP and SRB assays, and SRB and MTT assays 96 hours after the start of drug treatment. Results are expressed as mean and S.E.M. of at least two independent experiments.

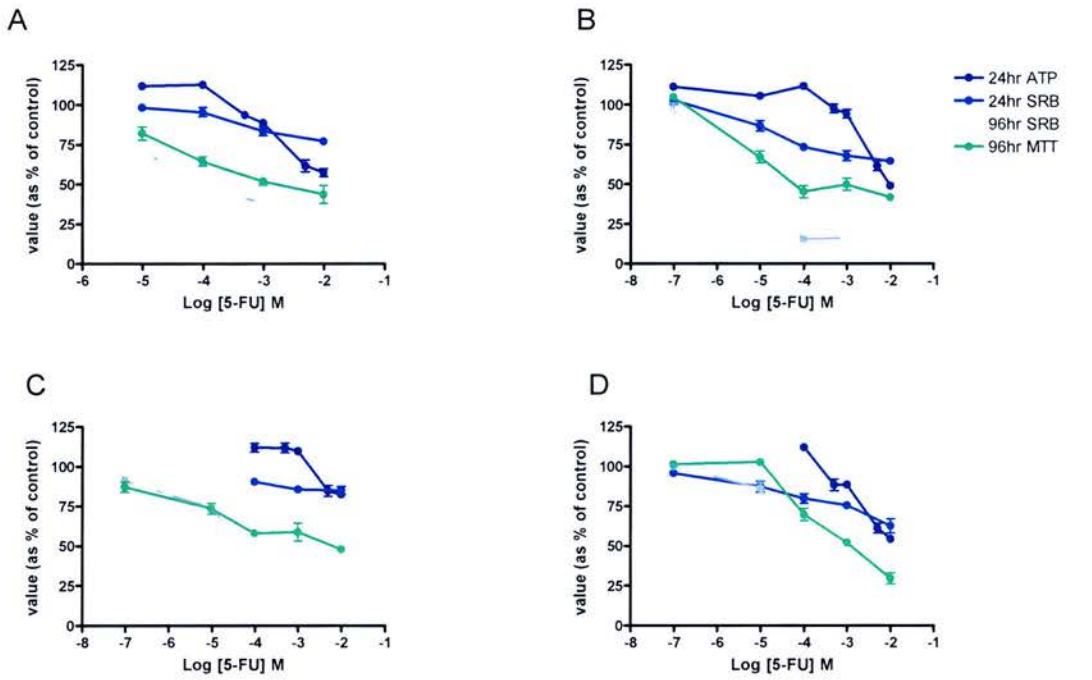


Figure 4.22: Comparison of ATP, SRB and MTT assay in hepatoma cell lines treated with 5-FU

Hep3B2.1-7 (A), Hep G2 (B), Huh-7D12 (C) and PLC/PRF/5 (D) cell lines were treated with a concentration range of 5-FU for 24 hours, followed by immediate ATP and SRB assays, and SRB and MTT assays 96 hours after the start of drug treatment. Results are expressed as mean and S.E.M. of at least two independent experiments.

In conclusion, the MTT, SRB and ATP assays show the same pattern of dose-dependent cell death with differences in decline with increasing drug concentration. It is not possible to determine if the IC_{50} values determined after 96 hours correlate with IC_{50} values determined immediately following drug treatment because an IC_{50} is not always reached. However, the ATP level in cells at 24 hours was plotted against the MTT and SRB assay data obtained after 96 hours for correlation analysis. When observing these data for the 4 hepatoma cell lines and fresh hepatocytes from 2 donors, no significant correlation ($p \leq 0.008$ to correct for multiple testing) was found for any of the drugs or cells using a Spearman correlation. Therefore it would not be suitable to substitute the ATP assay at 24 hours for the SRB or MTT assay at 96 hours.

4.6 DISCUSSION

The cell models considered, for the prediction of hepatotoxicity, respond to increasing concentrations of the three drugs investigated with varying levels of ATP loss and caspase-3/7 activation. The investigation of these models was intended to provide further insight into the mechanism of cell death and to assess if it was possible to detect hepatotoxicity by considering changes in cellular ATP and caspase activation earlier after drug exposure than the previously tested cytotoxicity assays.

The concept of using a variety of *in vitro* models to assess the effects of drugs on cells has been considered previously, but never to specifically assess hepatotoxicity of anticancer agents. For example, a variety of assays were investigated by Unilever to determine which was most suitable for detecting cytotoxicity in HepG2 cells using four compounds known to have cytotoxic activity (DMSO, butyric acid, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone and camptothecin) (Miret et al, 2006). This research indicated that a combination of assays was the best approach to evaluate the potential cytotoxicity of a compound and for the compounds they investigated in HepG2 cells they found ViaLight Plus (that measures intracellular ATP), ToxiLight (that measures cellular necrosis) and Caspsase-3 fluorometric assay resulted in the most useful combination. Similarly, research carried out by Promega demonstrated that the use of different assays to detect cell viability (including an ATP assay), cytotoxicity, and apoptosis (including a caspase activity assay) in HepG2 cells treated with tamoxifen and HL-60 cells treated with vinblastine provided an indication of the mechanism of cell death and they state that the choice of assay method is important (Riss and Moravec, 2004). The advantages and drawbacks of the two assays selected for this thesis are discussed below.

4.6.1 Adenylate Nucleotide Ratio Assay and Results

It has been described in publications and in the commercially available Apoglow™ assay from Cambrex that determination of ADP:ATP ratio allows discrimination between apoptosis and oncosis. Bradbury et al reported ADP:ATP ratios of 0.1 in control cells, of 0.11-1.0 in cells dying by apoptosis and of >15 in cells dying via oncosis. Contrary to results reported by Bradbury et al (2000), the use of the

adenylate nucleotide ratio assay in these studies did not prove a useful parameter to define the response to the three drugs tested here in terms of discriminating between oncosis and apoptosis. Indeed the results obtained by Bradbury et al were reproduced to an extent, where heat shock resulted in an ADP:ATP ratio substantially greater than 1, however ATP is completely depleted and is responsible for the higher ADP:ATP ratio observed: ADP levels remained lower in heat-shocked cells than in the controls which does not indicate oncosis despite the ADP:ATP ratio being high. Following none of the drug treatments where apoptosis was indicated as the mechanism of cell death using the caspase-3/7 assay in these studies was the ADP:ATP ratio consistent or interpretable, contrary to findings observed by Bradbury et al.

Therefore, in this thesis, ATP levels only have been presented. ATP depletion results in cell death and has been investigated as a therapeutic strategy for the treatment of cancer (Martin et al, 2000). It has been reported by Leist et al (1997) that in Jurkat cells treated with 1.2μM of the apoptosis-inducer staurosporin, ATP depletion >50% was sufficient to change the mode of cell death from apoptosis to necrosis, whereas higher ATP concentrations favoured apoptosis, and ATP loss ≥70% invariably resulted in oncosis. It is well known that the availability of ATP is a requirement for apoptotic cell death and this report suggests that cells can die via apoptosis up until they have lost 70% of their ATP after which oncosis would occur. Based on this fact, it is possible to assess in the studies presented in this thesis, where ATP levels are measured immediately following drug treatment, whether apoptosis or oncosis is likely occurring based on ATP levels. When cells were treated with mithramycin, none of the cells reached ATP levels of below 50% with the exception of the cryopreserved hepatocytes from donor C3 suggesting that these cells may be dying via apoptosis, in agreement with the caspase-3/7 data. Following oxaliplatin treatment, ATP levels reached approximately 50% when compared with control cells in the majority of models at the highest drug concentration investigated (250μM), again with the exception of cryopreserved hepatocytes (donors C2 and C3), again in agreement with the caspase-3/7 data. Finally, in 5-FU-treated cells ATP levels also reached approximately 50% when compared with control cells at the highest drug

concentration tested (10mM) in 3 of the hepatoma cell lines and in fresh hepatocytes from donor 13 and cryopreserved hepatocytes from donor C3, and in the other cells was higher than this. Again this would suggest that cells could die via apoptosis however less caspase activation was observed in cells (with the exception of fresh hepatocytes from donor 12) treated with 5-FU than for any of the other drugs.

4.6.2 Caspase-3/7 Assay and Results

The caspase-3/7 assay was used to detect caspase activity following treatment with mithramycin, oxaliplatin and 5-FU and was found to vary between the drugs and cell models. There have been isolated reports previously of the study of basal caspase activity and cell death mechanism in some of the cell models considered in these studies, and these are described below for comparison.

Caspase-3 was found to be over-expressed in hepatoma cell lines PLC/PRF/5, HepG2 and Hep3B2.1-7 compared to non-tumour liver tissue using Western Blot and immunohistochemistry techniques (Persad et al, 2004). However, only in PLC/PRF/5 and HepG2 did this result in an increased basal caspase-3 activity compared to SK-HEP-1, Hep3B and non-tumour liver tissue. Whilst PLC/PRF/5 and HepG2 are reported to have higher basal caspase-3 activity, there was no pattern detected in response to the three drugs considered in these studies and the response in terms of caspase activation in PLC/PRF/5 and HepG2 was dissimilar after treatment with mithramycin, oxaliplatin and 5-FU.

5-FU treatment (100μg/ml) for 24hr has been reported to induce ~10% apoptosis in Hep3B cells (Ganten et al, 2004). Similarly, in HepG2 cells treated with 100μg/ml 5-FU for 24hours, before identification of apoptotic cells by staining nuclear chromatin with Hoechst 33342, less than 10% apoptosis was observed (Meurrette et al 2005). At lower 5-FU concentrations (1μg/ml to 25μg/ml) HepG2 appeared more sensitive than Hep3B to undergo apoptosis (Jiang et al, 1999). Similarly, when caspase-3/7 activity was measured following 24-hour 5-FU treatment (10μM to 10000μM 5-FU) in these studies, only modest caspase activation was observed in

any of the cell lines and the maximum response in HepG2 cells (approximately 300% of control) was seen at the highest drug concentration tested (10mM).

Leroy et al, 2006 treated Jurkat and KG1a cells with mithramycin A and found that mithramycin at high concentrations induces apoptosis in these cells. Cells were treated for 48hours (in comparison with the 24hour incubation in this thesis) at concentrations ranging from 10-100nM and 100-500nM for Jurkat and KG1a cells respectively. Percentage of apoptosis was evaluated by DAPI staining (a method used to detect morphological changes) and reached approximately 30% apoptotic cells in both cell lines at the maximum drug concentration tested. Leroy et al, 2006 demonstrate that mithramycin can induce the Fas apoptotic pathway in Jurkat and KG1a cells and speculate that mithramycin induces the Fas apoptotic pathway in hepatocytes which may in part explain the hepatotoxicity of this drug. Caspase activation activity was observed with increasing mithramycin concentrations particularly in Hep3B2.1-7, PLC/PRF/5 and in hepatocytes from donor 12.

4.6.3 Mechanisms of Cell Death in Hepatocyte Models

The drugs selected target replicating cells (e.g. 5-FU interferes with DNA synthesis) therefore it is likely that these drugs have other cellular targets in the liver which is a non-replicating organ under normal circumstances. The investigation of different models to assess liver toxicity was intended to allow understanding of the broad mechanism of cell death induced by mithramycin, oxaliplatin and 5-FU (i.e. oncosis or apoptosis) in the different models and should help assess which *in vitro* models respond in the same way as the fresh human hepatocytes and the same as the human liver.

It should be noted that not all cells in a tumour will die by the same mechanism after exposure to a particular drug. In tumour and in liver, differences in the concentration of drug exposed to and in the cellular energy status will occur and are likely to result in differing cell death. The mechanism of cell death has been reported to differ according to cell density, and will be affected by concentration and duration of drug exposure therefore the conclusions presented herein are specific to the drug

treatments used in these studies and may be applicable to other situations using the same drugs.

4.6.4 Earlier Detection of Cell Death

The possibility of detecting hepatotoxicity earlier than was possible using the cytotoxicity assays presented in chapter 3 was investigated, by observing data obtained in cell models immediately following 24-hour drug treatment with three drugs. With the 96-hour SRB and MTT data that was determined previously, it was possible to detect the IC_{50} concentration in most cases. However, after treating cells over a similar concentration of the three drugs 24-hours after the start of drug treatment when the ATP assay was performed it was not possible to determine drug concentrations resulting in 50% ATP loss compared to control in the majority of cases. Therefore for several drug concentrations, the % ATP loss compared to control was plotted against the % cell survival (detected by MTT or SRB assay) obtained at 96-hours, to assess if there was a correlation between the two.

Using Spearman correlation analysis, no significant correlations were found between the 2 parameters for any of the drugs in any of the cell models. Therefore it was concluded that whilst the ATP assay is useful for providing insight into the mechanism of cell death that could occur following drug treatment, it is not possible to substitute the 96-hour SRB and MTT assays with an ATP assay at 24-hours.

4.6.5 Conclusions

In conclusion, the data presented in this chapter provides insight into the mechanism of cell death in several *in vitro* models of the liver treated with mithramycin, oxaliplatin or 5-FU. Due to the difference in response to the three drugs tested in these studies, particularly in terms of caspase activation in the fresh hepatocytes from 2 donors, it is difficult to ascertain the response of hepatocyte models to these drug treatments. Certainly, all three of the drugs studied caused substantial caspase activation and therefore apoptosis in some of the models studied: mithramycin induced caspase activity in Hep3B2.1-7, PLC/PRF/5 and fresh hepatocytes from donor 12, oxaliplatin in all four of the hepatoma cell lines investigated and in fresh

hepatocytes from donor 12, and 5-FU in HepG2 cells and fresh hepatocytes from donor 12. In order to further define the mechanism of response of fresh hepatocytes to the drug treatment, it would be useful to study hepatocytes from more donors. Due to the difference between the cell models in response to these drugs it has not been possible to ascertain which is the most appropriate model to use for assessment of hepatotoxicity. The ATP data obtained at the end of drug treatment for 24-hours did not correlate with the SRB or MTT data 96-hours following the start of drug treatment. On the basis of the data presented in these studies it would not be suitable to replace the 96-hour SRB or MTT assay with an earlier assay detecting changes in ATP or caspase-3/7 activity with drug treatment.

CHAPTER 5: Conclusions and future directions

5.1 SUMMARY AND CONCLUSIONS

Drug-induced hepatotoxicity is a major cause of failure of drug candidates during development and of drug withdrawal post-marketing. Exposure of individuals to certain anti-cancer agents can result in liver toxicity. Anti-cancer drugs induce cytotoxic effects in all proliferating cells and, to a certain extent, in non-dividing cells. For some anti-cancer drugs this manifests as liver-related dose-limiting toxicity. More toxicities are accepted with anti-cancer drugs than would be accepted with other classes of agents (e.g. drugs that cause hair loss or vomiting are routinely used in the treatment of cancer), however drugs that cause major liver toxicity cannot be used in the treatment of patients. Liver toxicity has been identified in early-phase clinical trials of anti-cancer agents and their clinical development stopped. Unfortunately, current pre-clinical models poorly predict a potentially hepatotoxic anti-cancer agent and an accurate and reproducible method for its detection would be beneficial.

Therefore the aim of the studies presented in this thesis was to evaluate *in vitro* models in which anti-cancer drug-induced hepatotoxicity may be detected, which may aid the incorporation of appropriate hepatotoxicity screening pre-clinically and decrease the likelihood of hepatotoxic anticancer agents entering clinical development. In addition, for agents where hepatotoxicity has been detected during clinical development and it is thought useful to study this toxicity *in vitro*, knowledge of the most relevant *in vitro* model would be valuable.

To determine whether hepatotoxicity caused by anticancer agents can be detected in *in vitro* models and find out which model may be the most appropriate, this study considered several *in vitro* models and assays using anti-cancer agents with known hepatotoxic or non-hepatotoxic properties in humans. The hypothesis was that isolated fresh hepatocytes *in vitro* would be the most indicative model of human hepatocytes *in vivo,* in which to detect toxicity. Due to the potential limitations

associated with the use of fresh hepatocytes, including limited source, inter-donor variability and relatively high cost, other models were also considered.

In vitro cell models of human origin were selected to represent hepatocytes: the predominant cell type in the liver. As described in chapter 2, fresh and cryopreserved human hepatocytes did not proliferate in culture and expressed several liver-specific genes, although expression was generally lower in cryopreserved- in than fresh- hepatocytes. Hepatoma cell lines and an immortalised hepatocyte cell line were also investigated, and these cells proliferated in culture and expressed lower levels of the liver-specific genes investigated than the fresh hepatocytes, with the exception of CYP1A1. These models therefore represent a spectrum of cell types with different properties, most of which have previously been used for investigation of hepatotoxicity *in vitro* with other classes of drugs. Based on these findings, fresh hepatocytes would be selected as the model most similar and therefore relevant to the human liver in that they have liver-specific gene expression and are non-proliferating.

The *in vitro* models were treated with a range of concentrations of three anti-cancer drugs (mithramycin, oxaliplatin and 5-FU) all of which have been used in the treatment of patients with cancer for several years and therefore the ability of these agents to cause hepatotoxicity in humans has been determined. As presented in chapter 3, in all the cell models, drug treatment resulted in dose-dependent cell death, as determined by SRB and MTT assays. By treating the *in vitro* models with a concentration range of each drug, an IC_{50} for each drug in each model was determined. The IC_{50} values determined in the different models varied considerably, and within the fresh hepatocytes inter-individual variability was also observed. By comparing the IC_{50} in each liver model (toxic) to the median IC_{50} in colon cancer cell lines (therapeutic), it was possible to define a 'therapeutic index' for each drug in each model. Based on these data, fresh hepatocytes, and the Hep3B2.1-7, Huh-7D12, PLC/PRF/5 and THLE-2 cell lines were able to discriminate the hepatotoxic drug mithramycin from the other 2 drugs investigated.

In addition to the studies carried out with mithramycin, oxaliplatin and 5-FU, investigations were carried out with the novel ruthenium compounds, RM175 and HC11 using fresh hepatocytes and hepatoma cell lines. The cytotoxicity data obtained and definition of a therapeutic index for these compounds suggested that both of these compounds would have the potential to cause hepatotoxicity, and in these models HC11 was found likely to be more hepatotoxic than RM175. These findings agree with *in vivo* studies in mice using RM175 and HC11 which described liver toxicity as being more severe with HC11 than RM175. If *in vitro* definition of the therapeutic index had been carried out early in drug development, it may have avoided the need to perform *in vivo* toxicity experiments.

ATP levels and caspase-3/7 activation was also investigated following drug treatment and the results presented in chapter 4. ATP levels were found to drop with increasing drug concentration in the majority of cell models investigated with the 3 drugs tested. Analysis of caspase-3/7 activity revealed that at the higher drug concentrations, for each drug tested, there was caspase-3/7 activation suggesting that apoptosis was occurring. However, no drug- or model-specific pattern of cell death was observed. The ATP data obtained at the end of drug treatment for 24-hours did not correlate with the SRB or MTT data 96-hours following the start of drug treatment. On the basis of the data presented in these studies it would not be suitable to replace the 96-hour SRB or MTT assays with an earlier assay detecting changes in ATP or caspase-3/7 activity with drug treatment.

In summary, considering the gene expression and cytotoxicity data together has revealed that there is no one model that would be ideal for the detection of hepatotoxicity *in vitro*, and that rather a combined approach using more than one cell model, knowing the limitations of each, is likely to provide the most useful information in future studies. Defining a therapeutic index as described in chapter 3 could be used as an approach to investigate hepatotoxicity pre-clinically, but earlier detection of cell death using an ATP or caspase-3/7 assay would not be suitable for detecting the same hepatotoxicity at an earlier timepoint. Using fresh hepatocytes from at least 3 donors, 1 or more hepatoma cell line and the THLE-2 cell line would

be recommended based on the findings presented here. The cryopreserved hepatocytes and SK-HEP-1 cells should be excluded from analysis of hepatotoxicity in the way that is presented in this thesis: based on the gene expression analysis and cytotoxicity data it is possible to rule out SK-HEP-1 as a relevant model in which to study anti-cancer drug induced liver toxicity, and because the cryopreserved hepatocytes are damaged following cryopreservation and thawing, they too have been excluded. These data suggest that fresh hepatocytes are a suitable *in vitro* model in which to accurately identify hepatotoxicity associated with anti-cancer drugs: they express liver-specific genes, and are more sensitive to the hepatotoxic drug mithramycin, than to oxaliplatin and 5-FU. However, it was noted that the response of these cells from different donors was very variable. The fresh hepatocytes to be used for *in vitro* assays, based on the findings presented in chapter 2, should be transported in UW media to maintain cell viability. The hepatoma cell lines (Hep3B2.1-7, Huh-7D12, PLC/PRF/5) were also able to detect anti-cancer drug induced hepatotoxicity, however the toxic concentrations determined were far lower in these cell lines than in the fresh hepatocytes and they had lower expression of liver-related genes compared to the fresh hepatocytes. Based on the principal of defining a therapeutic index, THLE-2 cells were also able to discriminate mithramycin and could also be used for further investigations. More than one *in vitro* model should be used for future studies and a positive (e.g. mithramycin) and negative (e.g. cisplatin) control should be included in each experiment to ensure that the *in vitro* models respond as expected to known drugs and comparison between different compounds is possible.

5.2 FUTURE DIRECTIONS

The data presented here have provided a considerable contribution, investigating an appropriate model in which to study anti-cancer drug-induced hepatotoxicity. However, further studies could be undertaken, that would be valuable to expand the work presented here.

In terms of the models tested, hepatocytes were the cell type focused on due to their abundance in the liver and the key functions they have. When considering the gene

expression, fresh hepatocytes are the most similar to human liver, however as discussed there are problems associated with performing assays using fresh hepatocytes. There are several emerging systems that may provide practical advantages when compared with undertaking work with fresh human hepatocytes, including cryopreserved hepatocytes and immortalised cells. Cryopreserved hepatocytes are reportedly problematic to use for *in vitro* assays where attachment to a substratum and long periods of culture are required (Li et al, 1999) and this was confirmed in these studies. Viability loss during cryopreservation of hepatocytes is also a key concern, and in these studies cryopreservation resulted in few viable cells being available to work with. If alterations in the way the cells are cryopreserved or thawed could improve viability and attachment the use of cryopreserved human hepatocytes may increase. One promising approach to try and overcome some of these issues is the plating of fresh hepatocytes (and attachment to the substratum) before cryopreservation in a monolayer culture (Hengstler et al, 2000; personal communication with HannahCellScience). Cells are not commercially available that have been cryopreserved in this way, and was outwith the scope of this project, however if this technique does become available there is the likelihood that these may be useful for studies such as those presented here. A similar approach to that presented in chapter 3 could be investigated, treating the pre-plated cryopreserved hepatocytes over a concentration range of drugs with known hepatotoxic and non-hepatotoxic properties, to assess their use for the detection of hepatotoxicity. Furthermore, the possibility of immortalising hepatocytes, and other cell type of the liver could be considered to improve on the resources currently commercially available.

Several studies are also being carried out regarding the use of embryonic stem cells differentiated into many types of cells, one of which is hepatocytes (Cai et al, 2007). The exciting prospect of using functional hepatic cells generated from embryonic stem cells as a model in which to study hepatotoxicity is not yet possible. Once these cells become more readily available they may be able to overcome the source and supply problem encountered when fresh human hepatocytes are required.

In the studies presented in this thesis, models that represent hepatocytes have been investigated for the study of liver toxicity. One other system that may be of relevance to consider is that of co-cultures *in vitro*. As discussed, there are several other cell types in the liver (e.g. Kupffer cells, cholangiocytes), which can play a role in the response of the liver to certain toxic compounds. Co-cultures of hepatocytes with and without these cells may provide further information regarding the target cells of toxicity, and could be considered for *in vitro* assays.

The results presented focus on the assessment of hepatotoxicity by studying three anti-cancer drugs. Work was also undertaken to consider drugs that are currently undergoing pre-clinical and clinical development, but in order to build more confidence in the selected *in vitro* models, a larger group of anti-cancer compounds should be investigated. Continuing from this idea the *in vitro* liver models could be used to screen panels of drugs during the lead discovery phase of drug development. Comparing several lead compounds (selected on the basis of their desired activity), using *in vitro* hepatocyte models (e.g. fresh human hepatocytes, Huh-7D12, PLC/PRF/5, Hep3B2.1-7 and THLE-2) to define a therapeutic index could assess the hepatotoxic potential of each compound. This knowledge could aid in designing out hepatotoxic drugs: only the most promising candidate would be developed further based on both activity and toxicity considerations. This type of testing could be performed in addition to the multitude of other experiments that are carried out during drug development.

CHAPTER 6: Materials and Methods

6.1 MATERIALS
- 6.1.1 Cell Culture
- 6.1.2 Cytotoxicity Assays
- 6.1.3 RNA Extraction and qRT-PCR
- 6.1.4 Protein Isolation and Western Blotting

6.2 METHODS: CELL CULTURE
- 6.2.1 Maintenance of Cell Lines
- 6.2.2 Cell Harvesting
- 6.2.3 Cell Counting
- 6.2.4 Cryopreservation and Recovery of Cells from Liquid Nitrogen
- 6.2.5 Growth Characterisation of Proliferating Cell Lines
- 6.2.6 Fresh Hepatocyte Cell Culture
- 6.2.7 Counting & Assessment of Hepatocyte Viability by Trypan Blue Exclusion
- 6.2.8 Cryopreserved Human Hepatocyte Cell Culture

6.3 METHODS: *IN VITRO* STUDIES
- 6.3.1 Drugs
- 6.3.2 Sulforhodamine B (SRB) Assay
- 6.3.3 MTT Assay
- 6.3.4 Cytotoxicity Studies with Hepatocytes
- 6.3.5 Analysis of Cytotoxicity Data
- 6.3.6 Detection of the Mechanism of Cell Death

6.4 METHODS: RNA EXTRACTION AND qRT-PCR
- 6.4.1 RNA Extraction
- 6.4.2 DNase Treatment of RNA Samples
- 6.4.3 Quantification of RNA by NanoDrop
- 6.4.4 Assessment of RNA Integrity
- 6.4.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)
- 6.4.6 Size of qRT-PCR Products
- 6.4.7 Analysis of qRT-PCR data

6.5 METHODS: PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS
- 6.5.1 Protein Isolation from Tri Reagent
- 6.5.2 Bicinchoninic Acid Protein (BCA) Assay
- 6.5.3 Western Blotting

6.1 MATERIALS

Materials are listed under the appropriate technique, detailing the product and the supplier. All laboratory plastics (including tissue culture flasks, 96-well plates and cryovials) were from NUNC™ (via Fisher Scientific, Loughborough, UK), unless otherwise stated. All chemicals were from Sigma, Gillingham, UK unless otherwise stated.

6.1.1 Cell Culture

6.1.1.1 Primary Cells

Fresh and cryopreserved human hepatocytes were obtained from the United Kingdom Human Tissue Bank (UKHTB), and ethical approval was obtained from NHS Lothian (LREC/2003/8/42). Transport of these cells from UKHTB (Leicester, UK) to CRUK laboratory (Edinburgh, UK) involved car and aeroplane, and lasted in duration from 7 to 26 hours.

6.1.1.2 Cell Lines

Colon cancer cell lines (HCT 116 and SW620 [SW-620]) were obtained from American Type Culture Collection (ATCC; via LGC Promochem, Middlesex, UK), and HCT-8 [HRT-18], HCT-15, HT-29 and COLO 205 were obtained from European Collection of Cell Cultures (ECACC; Salisbury, UK). Hepatoma cell lines Huh-7D12, Hep G2 [HepG2], Hep 3B2.1-7 [Hep 3B; Hep-3B; Hep3B] and PLC/PRF/5 [Alexander cell line] were also obtained from ECACC, and SK-HEP-1 was from ATCC. The immortalised liver cell line THLE-2 was also from ATCC.

6.1.1.3 Cell Culture Materials

BD BioCoat™ Collagen I cellware	BD Biosciences, Oxford, UK
FNC coating mix®	Stratech Scientific Limited, Soham, UK
RPMI 1640 (+L-glutamine, +phenol red)	GIBCO, Invitrogen, Paisley, UK
DMEM (+1000mg/L glucose, +580mg/L L-glutamine, + 110mg/L pyruvate, + 15mg/L phenol red)	GIBCO, Invitrogen, Paisley, UK
Bronchial Epithelial Medium (BEBM®)	Cambrex, Wokingham, UK

William's Medium E (- phenol red)(WEM)	Cambrex, Wokingham, UK
University of Wisconsin (UW) solution	Bristol-Myers Squibb, New York, USA
Celsior® solution	Sangstat, Fremont, USA
Foetal calf serum (FCS)	Harlan Seralab, Loughborough, UK
Penicillin and streptomycin	GIBCO, Invitrogen, Paisley, UK
BEGM® singlequots	Cambrex, Wokingham, UK
Human epidermal growth factor (EGF)	Sigma, Gillingham, UK
O-phosphoethanolamine	Sigma, Gillingham, UK
Dexamethasone	VWR International, Lutterworth, UK
Insulin, bovine	GIBCO, Invitrogen, Paisley, UK
L-glutamine	Sigma, Gillingham, UK
Cell dissociation solution	Sigma, Gillingham, UK
Cell scraper	BD Biosciences, Oxford, UK
Phosphate buffered saline (PBS)	GIBCO, Invitrogen, Paisley, UK
Coulter Counter®	Beckman Coulter, High Wycombe, UK
Water bath	Grant Instruments Ltd, Cambridge, UK
Diavert microscope	Leitz, Wetzlar, Germany
Heraeus HERAcell® incubator	Thermo Scientific, Basingstoke, UK

6.1.2 Cytotoxicity Assays

Mithramycin	Sigma, Gillingham, UK
Oxaliplatin	Sanofi-aventis, Surrey, UK
5-fluorouracil (5-FU)	Sigma, Gillingham, UK
RM175	Peter Sadler, Edinburgh University, UK
HC11	Peter Sadler, Edinburgh University, UK
Recombinant human TRAIL/TNSF10	R&D Systems, Minneapolis, USA
Sulforhodamine B (SRB)	Sigma, Gillingham, UK
Thiazolyl blue tetrazolium bromide (MTT)	Sigma, Gillingham, UK
Dimethyl sulfoxide (DMSO)	VWR International, Lutterworth, UK
Trichloroacetic acid (TCA)	Sigma, Gillingham, UK
ApoONE® homogeneous caspase-3/7 assay	Promega, Southampton, UK
Purified Active Recombinant Human	

Caspase-3 (CPP32)	BD Biosciences, Oxford, UK
Purified Active Recombinant Human Caspase-7 (Mch3)	BD Biosciences, Oxford, UK
ApoGlow® Kit	Cambrex, Wokingham UK
CellTiter-Glo® Luminescent Assay	Promega, Southampton, UK
Microlumat plus LB 96V Luminometer	Berthold Technologies, Redbourn, UK
Fluoroskan® *Ascent* FL Fluorometer	Thermo Labsystems, Basingstoke, UK
Biohit BP800 microplate reader	Biohit, Helsinki, Finland
GraphPad Prism® software	Version 4.00 for Windows, San Diego, California, USA, www.graphpad.com

6.1.3 RNA Extraction and qRT-PCR

Tri® reagent	Sigma, Gillingham, UK
TURBO DNA-free™	Ambion, Huntingdon, UK
DNaseI, RNase-free	Roche, Mannheim, Germany
Protector RNase inhibitor	Roche, Mannheim, Germany
RNA 6000 Nano LabChip® kit	Agilent, Wokingham, UK
Agilent 2100 Bioanalyser	Agilent, Wokingham, UK
NanoDrop® ND-1000 Spectrophotometer	NanoDrop Tech., Delaware, USA
RT-PCR kit (SYBR® green system)	Qiagen Ltd, Crawley, UK
Sigma RT-PCR primers	SigmaGenosys, UK
CRUK RT-PCR primers	Oligo Synthesis Lab, Potters Bar, UK
Human liver total RNA (adult & foetal)	BD Biosciences, Oxford, UK
Rotorgene™ RG-3000	Corbett research, Cambridge, UK
RT-PCR analysis software	Corbett research version 6.1 (Build 25) www.corbettresearch.com
MJ Research PTC-225 thermal cycler	Thermo Labsystems, Basingstoke, UK
GelDoc-It™ Imaging System	UVP, Cambridge, UK
Heraeus® biofuge pico/fresco	Sorvall, Bishop's Stortford, UK
RC-6™ Superspeed Centrifuge	Sorvall, Bishop's Stortford, UK
RT-PCR tubes	Corbett Research, Cambridge, UK

6.1.4 Protein Isolation and Western Blotting

Bicinchoninic acid (BCA) protein assay	Sigma, Gillingham, UK
1mg/ml BSA protein standard	Sigma, Gillingham, UK
(Bis)Acrylamide solution (30%w/v)	Severn Biotech Ltd, Kidderminster, UK
TEMED	Bio-Rad laboratories, Hertfordshire, UK
Full-Range rainbow MW markers	Amersham Biosciences, Chalfont St Giles, UK
TECHNE: DRIBLOCK®DB-2A	VWR International, Lutterworth, UK
Electrophoresis and transfer tanks	Bio-Rad laboratories, Hertfordshire, UK
Power pack: Power PAC 3000	Bio-Rad laboratories, Hertfordshire, UK
Immobilon-P PVDF Membranes	Millipore, Watford, UK
Tween® 20	Bio-Rad laboratories, Hertfordshire, UK
Ponceau S	Sigma, Gillingham, UK
Western blotting detection kit	Santa Cruz Biotechnology, Inc, Santa Cruz, CA
Photographic paper: hyperfilm	Amersham Biosciences, Chalfont St Giles, UK
pH210 pH meter	Hanna Instruments, Leighton Buzzard, UK

6.2 METHODS: CELL CULTURE

All procedures were carried out at room temperature unless otherwise stated.

6.2.1 Maintenance of Cell Lines

All cell lines are of human origin, and further information regarding the cell lines can be found in table 6.1. All cells were grown in a humidified 37°C 5% CO_2 incubator, and were regularly tested for mycoplasma contamination and found to be negative. Penicillin and streptomycin (antibiotics) were aliquoted at concentrations of 10000units/ml and 10000µg/ml respectively and stored at -20°C. These were added to all tissue culture medium at a 1:100 dilution. Foetal calf serum (FCS) was heat-inactivated at 55°C, aliquoted, and stored at -20°C. Before use FCS was thawed in a 37°C water bath.

The colon cancer cell lines were grown as monolayers in RPMI (containing phenol red) supplemented with 5% heat-inactivated FCS, and 1% antibiotics. The hepatoma cell lines were grown as monolayers in DMEM (containing 1000mg/L D-glucose, L-glutamine, 110mg/L sodium pyruvate, and phenol red) and supplemented with 10% heat-inactivated FCS and 1% antibiotics. Cultures of hepatoma and colon cancer cell lines were split once per week, using cell dissociation solution, and the medium was changed once per week.

Immortalised hepatocytes were grown as monolayers in BEBM® supplemented with 10% heat-inactivated FCS, 1% antibiotics, BEGM® singlequots (Bovine pituitary extract, insulin, hydrocortisone, retinoic acid, transferrin, triiodothyronine, and human epidermal growth factor), 5ng/ml EGF and 70ng/ml O-phosphoethanolamine. Culture flasks and plates were pre-coated with FNC coating mix for growing the immortalised liver cell line, medium was changed every second day, and cultures were split when cells were 70-80% confluent using cell dissociation solution and a cell scraper.

6.2.2 Cell Harvesting

Proliferating cell lines were grown in monolayers until they reached 70-80% confluence, in 25, 75 or 175cm^2 tissue culture flasks. Media was removed from flasks and the monolayer washed with pre-warmed PBS, pH 7.3 to remove traces of serum that could affect dissociation of cells. 1-3mls of cell dissociation solution was then added to flasks at room temperature and left for 1-10 minutes at room temperature. Cell dissociation solution was inactivated by addition of serum-containing media, and cells were quickly pipetted up and down to remove any clumps of cells.

6.2.3 Cell Counting

To obtain the appropriate cell concentration for experimental set up, cells were counted on a Coulter Counter. Cells previously dissociated from the flask were resuspended in 15mls media and the suspension was then passed through a syringe to

ensure single cell suspension and accurate cell counts. Briefly, 200µl of the cell suspension was added to 9.8mls sterile sodium chloride (0.9%) and counted by the coulter counter. Cells were diluted appropriately for seeding and re-counted to confirm dilutions.

Table 6.1: Source and details of cell lines used for *in vitro* studies

CELL LINE	ESTABLISHED FROM	DONOR DEMOGRAPHICS	REFERENCES
COLO 205	Colon adenocarcinoma, ascites	Male, Caucasian, aged 70	Semple et al., 1978
HCT-8	Ileocecal colorectal adenocarcinoma	Male, aged 67	Rosenthal et al., 1977
HCT-15	Colorectal adenocarcinoma, Dukes' type C	Male	www.ecacc.org.uk
HCT 116	Colorectal adenocarcinoma	Male, adult	Brattain et al., 1981
HT-29	Colorectal adenocarcinoma	Female, Caucasian, aged 44	www.ecacc.org.uk
SW620	Colorectal adenocarcinoma, Dukes' type C, from lymph node	Male, Caucasian, aged 51	Stragand et al., 1981
Hep 3B2.1-7	Hepatocellular carcinoma	Male, Black, aged 8	Aden et al., 1979
Hep G2	Hepatocellular carcinoma	Male, Caucasian, aged 15	Aden et al., 1979
Huh-7D12	Hepatocellular carcinoma	Male, Japanese, aged 57	Nakabayashi et al., 1982
PLC/PRF/5	Hepatocellular carcinoma	Male, African	Alexander et al., 1976; Daemer et al, 1980.
SK-HEP-1	Liver ascites adenocarcinoma	Male, Caucasian, aged 52	Heffelfinger et al., 1992
THLE-2	Liver, left lobe, SV40 transformed	Adult	Pfeifer et al, 1993

6.2.4 Cryopreservation and Recovery of Cells from Liquid Nitrogen

Cells to be stored in liquid nitrogen were dissociated from flasks as above, and centrifuged for 5 minutes at 1800rpm (1000g). Supernatant was removed and the cell pellet resuspended in 10mls of freezing mix (10% DMSO in 90% FCS). 1ml of this solution was aliquoted into each labelled cryovial and frozen immediately to -70°C overnight before transfer into liquid nitrogen tank at approximately -180°C. Upon removal from liquid nitrogen, the cryovial was placed in a 37°C water bath for 1-2mins until thawed, and centrifuged for 5mins at 1000g to obtain a cell pellet. Supernatant was removed and the pellet was resuspended in pre-warmed medium. Cells were left overnight to attach to the flask, before media was changed to remove cell debris.

6.2.5 Growth Characterisation of Proliferating Cell Lines

To determine doubling times, plating efficiencies and seeding densities for cytotoxicity assays, cell growth curves were produced, using the SRB assay to provide a measure of cell number. Cells were plated in 96-well plates at increasing densities from 500 to 5000 cells/well. Each cell density was plated as 6 replicates in up to 7 plates, to produce a time-course over 7 days. Cells were also plated at increasing densities for the calibration plate (500 to 50000 cells/well). All cells were allowed to adhere overnight. Drug-treatment was mimicked 24 hours after plating the cells, by changing media in 3 rows (out of 6 replicates), which allowed assessment of plating efficiency. The calibration plate was fixed with trichloracetic acid (TCA) 24hours after plating to verify the linearity of SRB staining with cell density. One plate was fixed and quantified every 24 hours for 7 days according to the SRB assay protocol, and the results were used to produce cell growth curves.

For each cell line, a calibration curve and growth curves were produced. An example of the curves produced is shown in figures 6.1 and 6.2, using the hepatoma cell line HepG2. The calibration curve (figure 6.1) was produced by SRB staining, and is linear over a range of cell densities (500-50000cells/well, R^2=0.998). From the plates stopped every 24 hours for 7 days, growth curves were generated and can be

seen in figure 6.1. OD (optical density) increased over time at all cell densities except where 500 cells/well were plated. Using the calibration equation (determined from the calibration plate stopped on day two), optical density values from the growth curves were converted to cell numbers. Cell growth is an exponential process and the time taken for a population doubling can be calculated from the graph in figure 6.2. Assuming cells grow at an exponential rate, doubling times were calculated by plotting log cell number against time, and fitting the data with an exponential trend-line. The plating efficiency was calculated as a ratio between mean OD of wells that were washed to mimic drug treatment, and mean OD of unwashed wells.

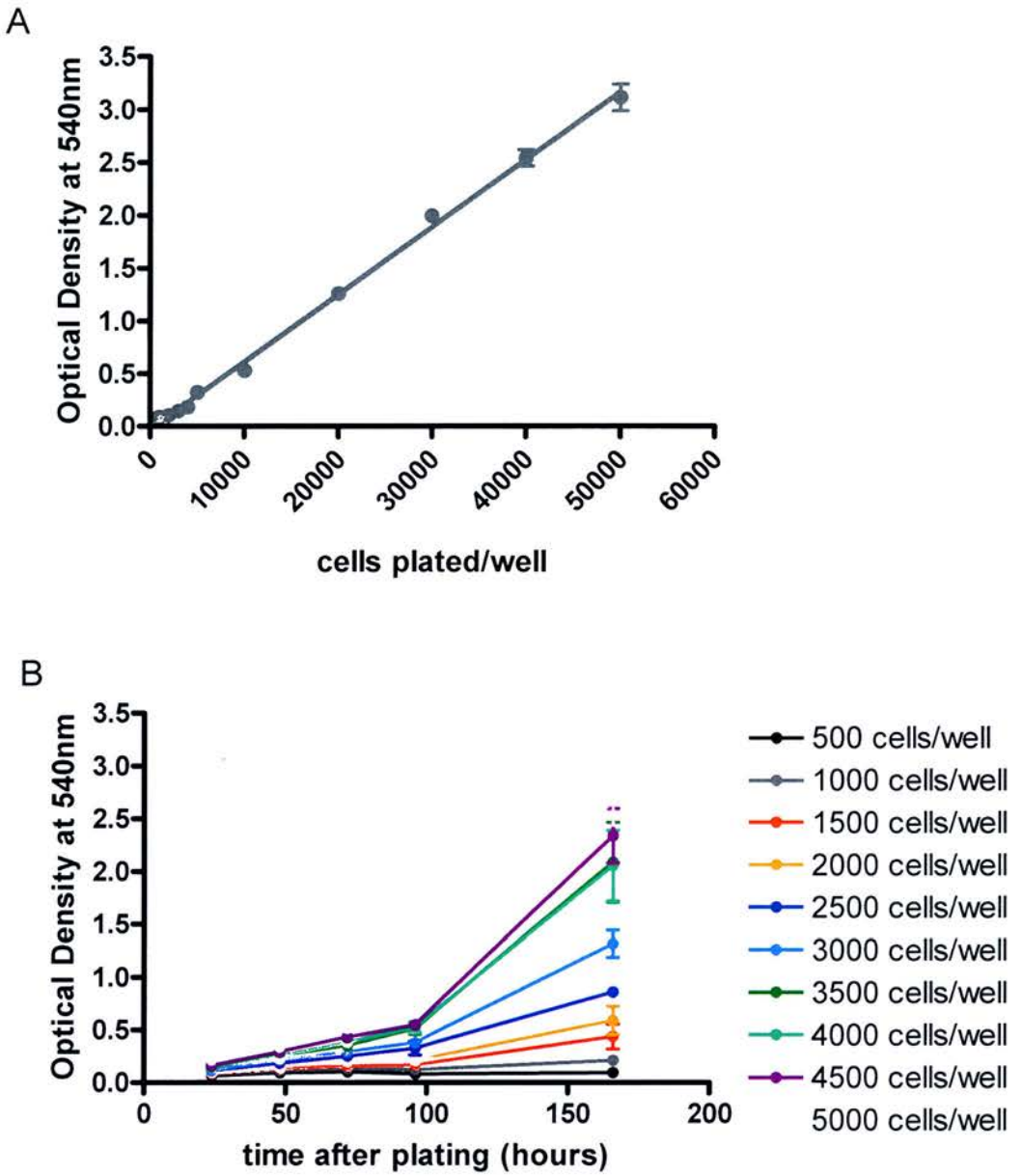


Figure 6.1: Calibration, and OD per density of HepG2 cells

HepG2 cells were plated at increasing cell densities in supplemented DMEM media in 96-well plates. The calibration plate was stopped on day 2 and number of cells plated was plotted against OD at 540nm (A). One day-growth plate was stopped per day and cellular protein was stained using the SRB assay (B).

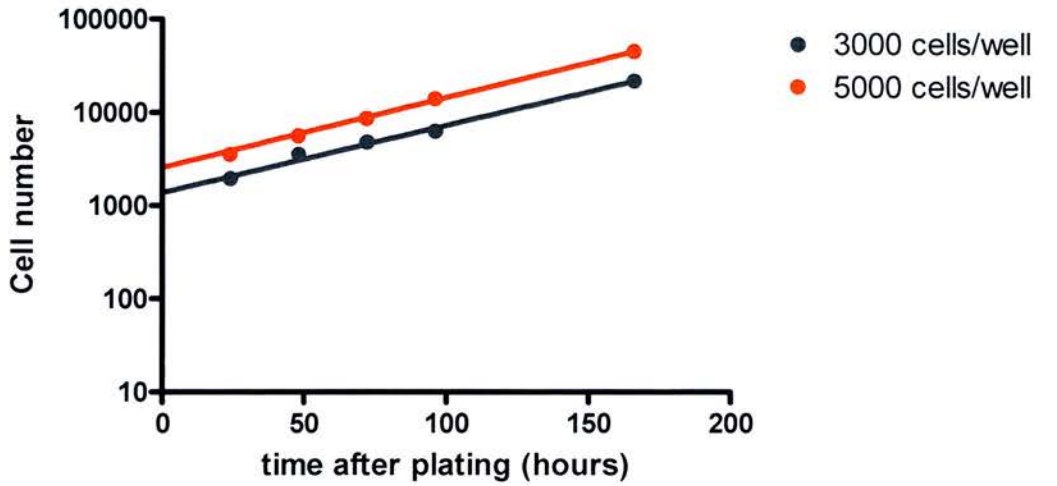


Figure 6.2: Exponential growth of HepG2 cells

Cells were plated in supplemented DMEM media in 96-well plates. The calibration plate was stopped on day two along with one day-growth plate, and then one day-growth plate per day afterwards. Cellular protein was stained using the SRB assay.

For comparison with cells in which the selected drugs are used to treat, some characterisation is required for use of colon cancer cell lines in experiments. The doubling times for the six colon cancer cell lines selected do not vary greatly from one another (range 20.9-27.3 hours), and can be seen in table 6.2. However the plating efficiency and time to reach confluence do vary, and result in different seeding densities for the cytotoxicity assays.

Table 6.2: Doubling time, plating efficiency & plating density of colon cancer cell lines

CELL LINE	CELL DOUBLING TIME AT OPTIMUM CELL DENSITY (HOURS)	PLATING EFFICIENCY (%)	OPTIMUM PLATING DENSITY FOR CYTOTOXICITY STUDIES (cells/well)
COLO 205	26.2	22	3000
HCT-8	21	93	1500
HCT-15	20.9	100	1500
HCT 116	22.3	97	1500
HT-29	27.3	110	2000
SW620	23.5	94	5000

6.2.6 Fresh Hepatocyte Cell Culture

Hepatocytes were isolated by the UKHTB by performing a 2-step collagenase digest method following the published method of Seglen (1976). The UKHTB usually receives liver tissue on ice in a kidney perfusion buffer from the surgery where isolation took place. Any open vessels are cannulated and perfused. A peristaltic pump with warming jackets (flow rate approximately 20 ml min) is used, and the liver is perfused with perfusion buffer (Invitrogen), and digested with digest buffer (Invitrogen). It is then minced with scissors to release hepatocytes, filtered through 100μm Nybolt gauze, and washed 3 times at 75g in modified DMEM. Isolated hepatocytes were then ready for distribution.

Cells were obtained from UKHTB in suspension (transported either in Celsior® solution, DMEM or University of Wisconsin solution) on melting ice. Cells to be used for cytotoxicity studies were always transported in DMEM supplemented with 2g/l human serum albumin. Contents of the tube were resuspended and centrifuged at 70g for 5minutes. The supernatant was aspirated and the pellet of hepatocytes resuspended in WEM pre-warmed to 37°C. Media was supplemented with 2mM L-glutamine, 1% antibiotics, 10nM insulin, and 30nM dexamethasone. Cells were counted and viability assessed (see section 6.2.7) and either plated for cytotoxicity testing (Donors 1-13), RNA assessment (Donors 1-13) or used for transport media testing (Donors T1-T11). For donor demographics for cells used for cytotoxicity testing *in vitro*, RNA extraction and for optimisation of transport media, please refer to tables 6.3 and 6.4 respectively. It was found by Hewes et al (2006) that prior treatment of patients with chemotherapy (5-FU ±oxaliplatin), whose hepatocytes were subsequently isolated, did not affect the viability or function of the isolated hepatocytes. Cell culture plates and flasks used for cells for cytotoxicity testing and RNA assessment were pre-coated with collagen I (BD BioCoat™). Cells did not proliferate in culture, however medium was changed every second day.

6.2.7 Counting & Assessment of Hepatocyte Viability by Trypan Blue Exclusion

Following centrifugation and re-suspension of hepatocytes in William's media E (WEM), cell viability was determined by trypan blue exclusion. Equal volumes of

stock hepatocyte solution and 0.4%(w/v) trypan blue (in PBS) were mixed and allowed to incubate for 3 mins at room temperature before counting of viable (white cells that excluded dye) and blue cells (compromised cells that took up the dye) in a haemocytometer. Briefly, cell suspension was placed on the haemocytometer before covering with cover slip and counting cells in appropriate chambers using a microscope.

$$\text{Cell viability (\%)} = \frac{\text{number of viable cells}}{\text{number of cells counted}} \times 100$$

6.2.8 Cryopreserved Human Hepatocyte Cell Culture

Frozen cells in freezing mix were received on dry ice from UKHTB and were transferred to a liquid nitrogen storage tank immediately upon arrival. Cryopreserved cells from 4 donors were investigated and the demographics of donors from which the hepatocytes were received can be seen in table 6.5. When required, vials of cryopreserved hepatocytes were removed from liquid nitrogen, and rapidly thawed in a 37°C water bath. Vials were then inverted gently to ensure contents were free of ice crystals (approximately 90seconds), before the contents of the vial were emptied into pre-warmed WEM and inverted to mix. The cells were then centrifuged at 40g for 5mins at 19°C, supernatant removed, and the pellet of cells was re-suspended in WEM heated to 37°C. The concentration and viability of cells was then determined as described in section 6.2.7 and cells plated immediately for cytotoxicity studies, or plated for 12hours before RNA extraction.

Table 6.3: Donor demographics of patients from which fresh hepatocytes were used for *in vitro* studies

SAMPLE I.D.	DATE	AGE	SEX	RACE	SMOKER?	CAUSE OF DEATH/ SURGERY	MEDICAL & DRUG HISTORY
DONOR 1 H3a, H2d	02/09/04	69	Female	Caucasian	Yes	Colorectal cancer	Asthma Hysterectomy Salbutamol, Beclometasone, terbinafine, On trovac trial
DONOR 2 H5a, H6d	07/10/04	62	Female	Caucasian	No	Colorectal metastases	Bowel resection
DONOR 3 H9a, H10d	01/12/04	54	Male	Caucasian	No	Colorectal metastases	Unknown
DONOR 4 H11a, H12d	20/01/05	36	Female	Unknown	Yes	Colorectal metastases	5-FU
DONOR 5 H13a, H14d	17/02/05	76	Male	Caucasian	No	Cerebro-vascular accident	Hypertension, transient ischemic attacks, Atenolol, Aspirin Amlodipine, In Intensive care: Noradrenaline, Amiodorone, piperacillin, tazobactam, Cefuroxime, metronidazole
DONOR 6 H15a, H16d	01/03/04	47	Female	Caucasian	Yes	Colorectal metastases	Bowel operation
DONOR 7 H17a, H18d	14/04/05	60	Female	Unknown	Yes	Liver resection	Unknown

SAMPLE I.D.	DATE	AGE	SEX	RACE	SMOKER?	CAUSE OF DEATH/ SURGERY	MEDICAL & DRUG HISTORY
DONOR 8*/** H19a, H20d	07/07/05	80	Male	Unknown	Yes	Colorectal metastases, gallbladder cancer	Dukes B tumour – bowel resection Jan 05 Gall bladder cancer – cholecystectomy Feb 05
DONOR 9** H21a, H22d	14/08/05	48	Female	Caucasian	Yes	Cholangiocarcinoma	Asthma – Salbutamol, beclometasone
DONOR 10 H23a, H24d	9/11/05	68	Female	Caucasian	Yes	Colorectal metastases	Bowel resection cancer (01/05) PEs, warfarin (05/05)
DONOR 11 H25a, H26d	14/12/05	61	Female	Caucasian	Yes	Colorectal metastases	Bowel resection
DONOR 12 H27a, H28d	28/06/06	58	Male	Unknown	No	Metastatic liver disease	Anterior Resection (05/06)
DONOR 13 H29a, H30d	15/08/06	63	Female	Caucasian	Yes	Cholangiocarcinoma	Hypertension, Ischaemic heart disease. Atenolol, aspirin, Questran

* Cryopreserved hepatocytes were also obtained from this donor

** Cells for transport media testing were also received from these donors

Table 6.4: Donor demographics from which hepatocytes were used for transport media testing

SAMPLE I.D. (RNA sample code)	DATE	AGE	SEX	RACE	SMOKER?	CAUSE OF DEATH/ SURGERY	MEDICAL & DRUG HISTORY
DONOR T1 Ht1-Ht4	28/05/05	73	Male	Caucasian	Yes	Colorectal cancer	Sigmoid colectomy and chemo 2003, no recent chemo
DONOR T2 Ht5-Ht8	04/06/05	57	Female	Caucasian	Yes	Colorectal metastases	Unknown
DONOR T3 Ht9-Ht12	07/07/05	80	Male	Unknown	Yes	Colorectal metastases, gallbladder cancer	Dukes B tumour – bowel resection Jan 05 Gall bladder cancer – cholecystectomy Feb 05
DONOR T4 Ht13- Ht16	19/07/05	54	Female	Caucasian	No	Colorectal cancer	Epilepsy –sodium valproate
DONOR T5 Ht17- Ht20	14/08/05	48	Female	Caucasian	Yes	Cholangiocarcinoma	Asthma – Salbutamol, beclometasone
DONOR T6 Ht21- Ht24	17/08/05	58	Female	Caucasian	Yes	Colorectal metastases	Unknown
DONOR T7 Ht25- Ht28	11/10/05	37	Female	Caucasian	No	Subarachnoid haemorrhage	Renal transplant 1984, failed in 1989, dialysis to present, hypertensive, 2 abnormal smears 2005
DONOR T8 Ht29- Ht32	17/10/05	32	Female	Caucasian	Yes	Subarachnoid haemorrhage	Unknown
DONOR T9 Ht33- Ht36	17/11/05	19	Female	Caucasian	No	Botryoid rhabdomyosarcoma of liver	Laparoscopic cholecystectomy

SAMPLE I.D. (RNA sample code)	DATE	AGE	SEX	RACE	SMOKER?	CAUSE OF DEATH/ SURGERY	MEDICAL & DRUG HISTORY
DONOR T10 Ht39- Ht42	24/11/05	52	Male	Caucasian	Yes	Liver resection for colorectal metastases	Colorectal resection 05, 5-FU
DONOR T11 Ht45- Ht48	15/12/05	68	Male	Caucasian	Yes	Liver metastases	Liver cancer, warfarin, omeprazole, penicillin

Table 6.5: Donor demographics of patients from which cryopreserved hepatocytes were obtained

SAMPLE I.D.	DATE	AGE	SEX	RACE	SMOKER?	CAUSE OF DEATH/ SURGERY	MEDICAL & DRUG HISTORY
DONOR C1 CRYO# 5354	04/06/05	57	Female	Caucasian	Yes	Colorectal metastases	Unknown
DONOR C2* CRYO# 5460	19/07/05	54	Female	Caucasian	No	Colorectal cancer	Epilepsy, Sodium valproate
DONOR C3* CRYO# 5514	28/05/05	73	Male	Caucasian	Yes	Colorectal cancer	Sigmoid colectomy and chemotherapy 2003
DONOR C4* CRYO# 5654	07/07/05	80	Male	Unknown	Yes	Colorectal metastases, gallbladder cancer	Dukes B tumour – bowel resection Jan 05 Gall bladder cancer – cholecystectomy Feb 05

*Cryopreserved cells from these donors were used for cytotoxicity assays

6.3 METHODS: *IN VITRO* STUDIES

A selection of *in vitro* assays were used to detect cell death or initiation of cell death in drug-treated cells. For all assays, cells were treated over a range of drug concentrations for each drug to be studied for 24hours before detection of cell death. Cytotoxicity studies for which cell death was considered as the endpoint were performed using the sulforhodamine B (SRB) assay and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Levels of ATP and caspase activity in cells were also measured following drug treatment.

6.3.1 Drugs

Stock solutions of mithramycin, oxaliplatin, and 5-fluorouracil were prepared using sterile water. Mithramycin was prepared as 0.5mg/ml (460.7μM) stock solution, and aliquots were stored at -80°C. Stock solutions of oxaliplatin and 5-FU were prepared fresh before use at concentrations of 1mg/ml (2.519mM) and 10mg/ml (76.9mM) respectively. The ruthenium compounds RM175 and HC11 have poor aqueous solubility and were therefore made up in a 5% DMSO (v/v) solution fresh before use at a concentration 10-times higher than the top concentration required to treat the cells. Kahalalide F was also prepared in a 5% DMSO solution at a concentration of 2mM fresh before use, and the top dilution was also always at least 10 fold lower than the stock concentration. TRAIL was reconstituted in sterile PBS containing 0.1% bovine serum albumin to a stock concentration of 20 μg/ml as per manufacturer's instructions. Aliquots of TRAIL were stored at -20°C and thawed before use. The appropriate TRAIL concentration was made up in the cell culture media for the appropriate cell line fresh before use.

6.3.2 Sulforhodamine B (SRB) Assay

The SRB assay is a colorimetric assay using the staining of cellular protein by SRB as a measure of cell number in 96-well plates (Skehan et al, 1990). Cells for staining with SRB were fixed to the plate by the addition of 50% (w/v) ice-cold TCA per well (250μl final volume) and incubated at 4°C for one hour. Wells were washed ten times with tap water, and air-dried. TCA–fixed cells were then stained with 50μl 0.4% (w/v) SRB solution (in 1% acetic acid (v/v)) at room temperature for 30

minutes. Unbound dye was then removed by 4 washes with 1% (v/v) acetic acid, and the plates were air-dried. Cellular-protein-bound SRB dye was then dissolved in 150µl 10mM Tris buffer per well (pH 10.5), and optical density (proportional to the cellular protein present), was measured at 540nm using a Biohit BP800 microplate reader. It can be seen from figure 6.1 that SRB staining is linear with cell density from 1000 to 50000 cells/well when HepG2 is used as an example, however this is true for all cell lines investigated.

6.3.3 MTT Assay

The MTT assay is based on the ability of viable cells to reduce a yellow tetrazolium compound to a blue formazan product (Banasiak et al, 1999). This conversion indicates metabolic activity of mitochondrial dehydrogenases in living cells. In the wells containing cells for MTT assessment, medium was removed and replaced with 200µl fresh medium. Cells were incubated for 3 hours in the dark with 50µl MTT solution, at 37°C. Medium was then removed, and formazan crystals were solubilised in 200µl DMSO and the optical density was measured at 570nm using a Biohit BP800 microplate reader. The concentration of MTT was optimised and it can be seen from figure 6.3 that MTT conversion is linear with cell density from 500 to 30000 cells/well when HCT116 is used as an example.

6.3.3.1 Determination of optimal MTT concentration

The HCT116 cell line was seeded over a range of cell densities (500-500000 cells/well) and allowed to attach for 24 hours. MTT was then added over a range of concentrations (1mg/ml to 6mg/ml), left to incubate for 3 hours in the dark, and the plate was read at 570nm. An MTT concentration of 2mg/ml was selected for future cytotoxicity studies with the MTT assay as no increase in OD values was obtained by increasing the MTT concentration from 2mg to a maximum of 6mg/ml, and conversion of 2mg/ml by HCT116 cells was linear over the range of cell densities investigated (R^2=0.985).

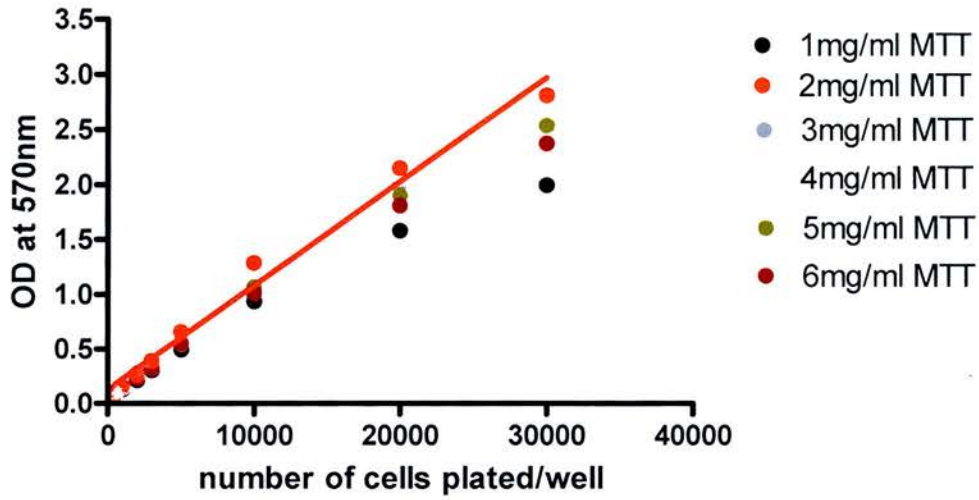


Figure 6.3: Determination of optimal MTT concentration for cytotoxicity assays

HCT116 cells were exposed to increasing concentrations of MTT (1mg/ml to 6mg/ml) for 3 hours, 24hours after plating, to determine the optimal concentration for cytotoxicity assays.

6.3.4 Cytotoxicity Studies with Hepatocytes

6.3.4.1 Fresh hepatocytes

Cells were obtained in suspension and assessed for viability using a trypan blue exclusion test. Cells (20000 viable/well) were plated in collagen-I pre-coated 96-well plates in supplemented WEM, and allowed to adhere for 12 hours at 37°C in a 5% CO_2 humidified incubator. Cells were then treated with a series of drug dilutions for 24-hours. At the end of drug exposure, the media was aspirated and the cells washed with 50µl PBS before adding 200µl/well of fresh complete WEM. Cytotoxicity studies were performed using the MTT and SRB assays.

6.3.4.1 Cryopreserved hepatocytes

Cryopreserved hepatocytes were thawed and viability was determined, as described. Cells (20000 viable/well) were plated in collagen-I pre-coated 96-well plates in supplemented WEM, and allowed to adhere for the minimum number or hours required for attachment (5 hours) at 37°C in a 5% CO_2 humidified incubator. Cells were then treated with a series of drug dilutions for 24-hours. At the end of drug exposure, the media was aspirated and the cells washed with 50µl PBS before adding 200µl/well of fresh complete WEM. Cytotoxicity studies were performed using the MTT and SRB assays.

6.3.5 Analysis of Cytotoxicity Data

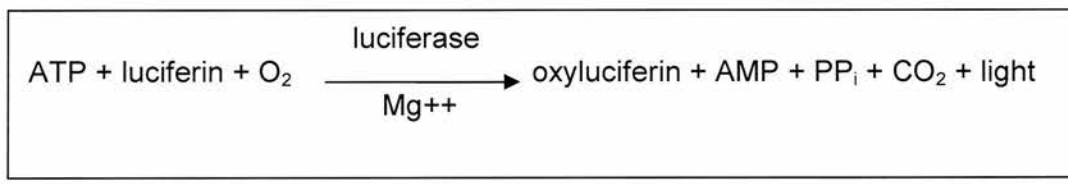
After treating cells over a concentration range for each drug it is possible to produce dose response curves. A sigmoidal dose-response curve with variable slope model was used to fit the data using GraphPad Prism Software, allowing analysis of data and determination of the IC_{50} values. IC_{50} values were defined as the concentrations that correspond to a reduction of cellular growth by 50% when compared with values of untreated control cells, or 50% cell death in the case of non-proliferating cells. The EC_{50} defined by GraphPad Prism represents the concentration of drug required to produce 50% of the maximal drug effect, therefore to determine IC_{50} it is necessary to read from the curve created. In this thesis it is the IC_{50} and not the EC_{50} concentrations that are considered, as it can be compared between cell lines and is not influenced by the maximum drug effect.

6.3.6 Detection of the Mechanism of Cell Death

Cells were plated in luminometer-grade 96-well plates (white polystyrene with clear bottoms) and allowed to attach to plate (24 hours for cell lines, 12 hours for fresh hepatocytes, 5 hours for cryopreserved hepatocytes). Cells were drug-treated for 24hours, and after removal of drug the ApoGlow® Assay and the Apo-ONE® homogeneous caspase-3/7 assay was performed at 24, 48 and 72-hours after the start of drug treatment.

6.3.6.1 ATP and ADP Assays

The adenylate nucleotide ratio assay (ApoGlow® assay, Cambrex) allows bioluminescent detection of both ATP and ADP levels in cell *in vitro*. This method utilises the enzyme luciferase, which catalyses the formation of light from ATP and luciferin according to the following reaction:



Where: ATP= adenosine triphosphate, O_2= oxygen, AMP= adenosine monophosphate, PP_i= pyrophosphate, CO_2= carbon dioxide.

To assess if ATP and ADP content is linearly related to cell number, standard curves were produced (figure 6.4). Both ATP and ADP are linearly related to cell number and over the range of 10-50000cell/well ATP had and R^2 of 0.999 and ADP had an R^2 of 0.982.

The ApoGlow™ assay comprises three successive measurements, detecting both ADP and ATP in cells. Firstly, Nucleotide Monitoring Reagent (NMR) and ADP-Converting Reagent (ADP-CR) were reconstituted in Tris Acetate buffer for a luminometer with 50µl volume injectors. Briefly, 100µl/well of nucleotide releasing reagent was added and after 5 minutes 50µl/well NMR (containing luciferin and luciferase) was added and an immediate reading taken (reading A) using a

luminometric plate-reader. The emitted light intensity measured in reading A is linearly related to ATP concentration in the cells. A second reading is taken after 40 minutes during which time the luminescent signal from the first reading decays (reading B). The ADP-CR (50µl/well) is then added to convert the ADP present to ATP and the luminescent signal was measured 5 minutes later (reading C). This provides ADP levels after subtracting the background reading (reading B). The ratio of ADP:ATP was calculated as [(reading C - reading B)/reading A)]. The kinetics of the ApoGlow™ assay can be seen in figure 6.5. The kit was used according to manufacturer's instructions with the exception of taking the ATP decay reading (reading B) after 40 minutes, instead of after 10 minutes.

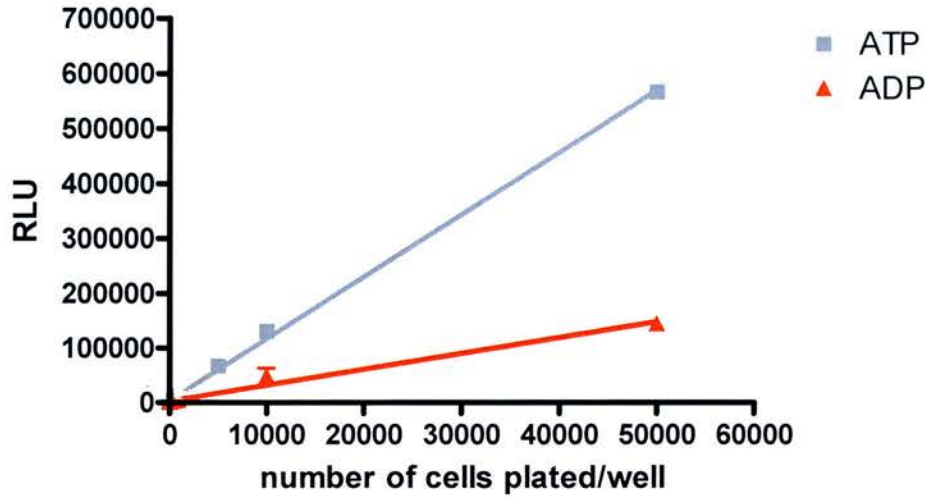


Figure 6.4: ATP and ADP Standard Curve

HCT116 cells were plated at increasing cell densities (10-50000 cells/well). ApoGlow™ assay was performed as described in section 6.3.6.1 and shows that ATP and ADP are linearly related to cell number.

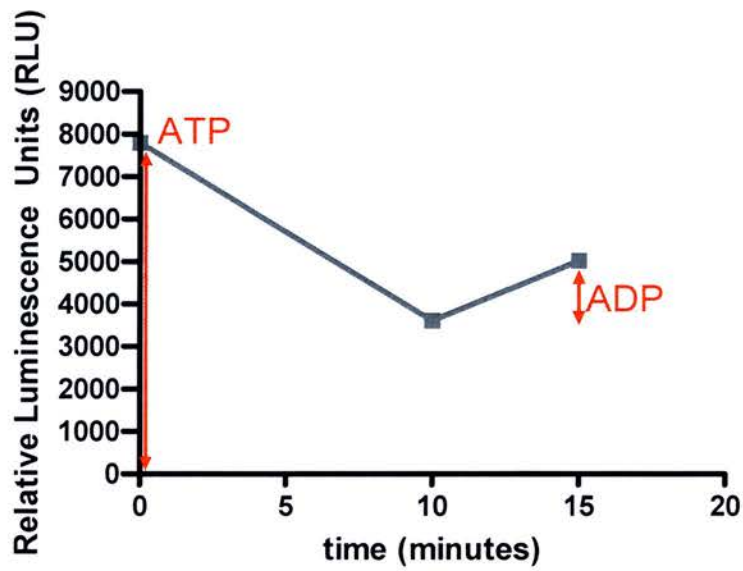


Figure 6.5: Kinetics of the ApoGlow™ assay

5000 HepG2 cells/well were plated 24hours before the assay. Cells were incubated with media to mimic drug treatment for 24hours, followed by the ApoGlow™ assay performed as described in section 6.3.6.1.

The use of the ApoGlow™ assay resulted in a large variability between samples and cell lines (data not shown). This is due to the continuous decay of the luminescent signal after the first measurement. The ADP measurements were therefore inaccurate, and resulted in ADP:ATP ratios that were not interpretable using the ApoGlow™ assay. A second ATP assay was therefore tested (CellTiter-Glo® Luminescent Assay, Promega). Although the principle of the assay is similar, the luminescent signal measuring ATP levels was more stable (Figure 6.6). After addition of the ADP converting reagent, the variability of the ADP measurements was reduced and both ATP and ADP levels were measured accurately.

It is clear that the half-life of the luminescent signal in the kit from Cambrex is far shorter (<40minutes) than that of the kit from Promega (greater than 6 hours). Because of the quick succession in which the readings are taken, CellTiter-Glo® reagent from Promega was selected to determine ATP levels followed by measurement of ADP (by conversion of ADP to ATP) using ADP-CR from Cambrex. Even using this method, reliable ADP measurements were not obtained and therefore ATP data alone is presented in chapter 4.

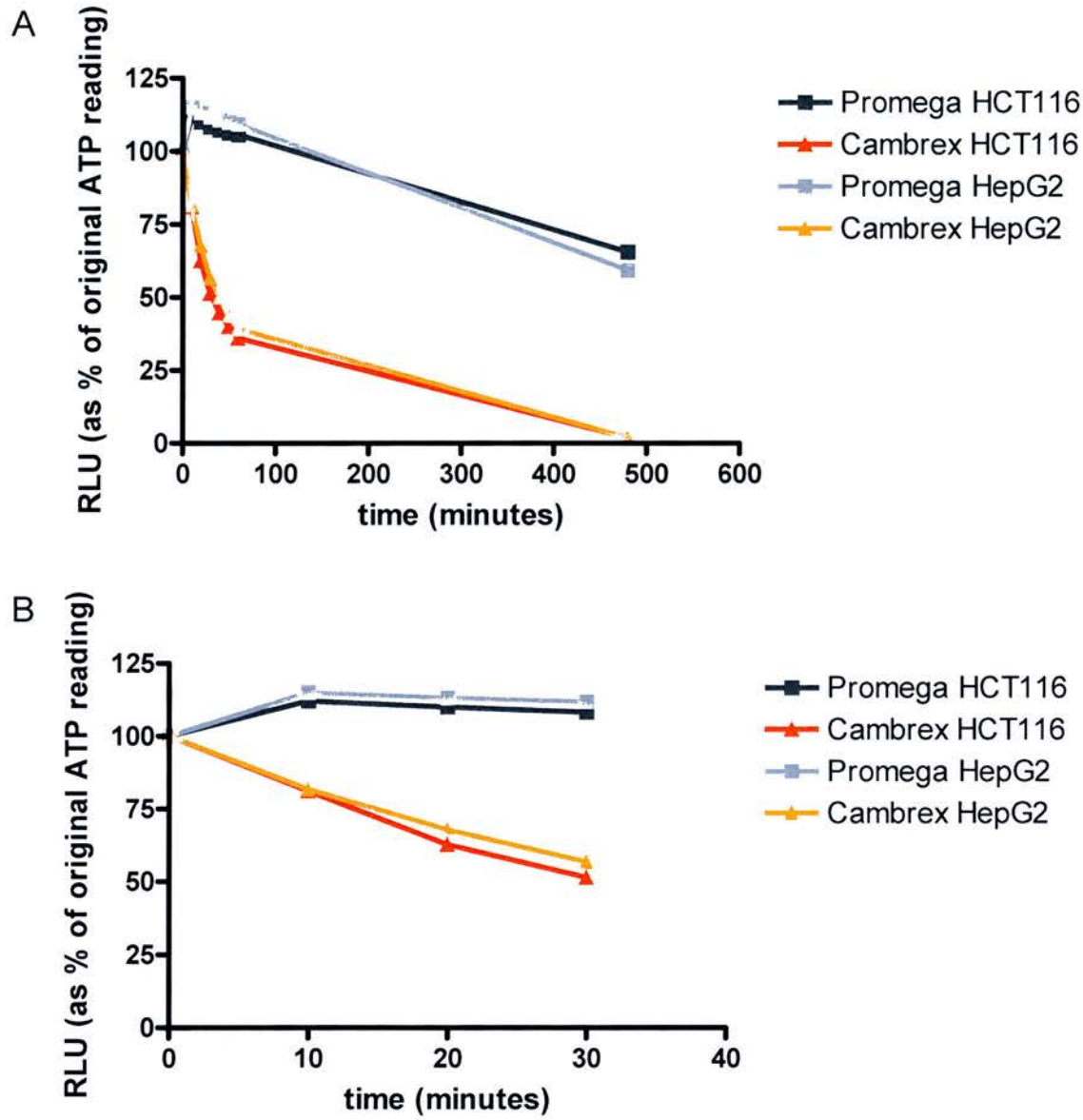


Figure 6.6: Comparison of Cambrex and Promega kit signal half-life

24-hours after plating (25000 HCT116/well and 13000 HepG2/well), cells were incubated with media to mimic drug treatment for 24hours, followed by addition of NRR and NMR (Cambrex), or CellTiter-Glo® reagent (Promega). ATP measurements were made immediately on the luminometer, and every 10 minutes thereafter for 1 hour, and then 6hours after the initial reading (A). B, shows the readings until 30minutes after initial reading as these are within the timeframe of the assay. Results are expressed as mean of duplicates, as a percentage of the initial ATP reading made.

6.3.6.2 Caspase-3/7 Activity Assay

The Apo-ONE® homogeneous caspase-3/7 assay allows measurement of caspase-3 and –7 activity, by measuring a fluorescent product produced by caspase-3/7 cleavage of a profluorescent substrate rhodamine 110, bis- (N-CBZ-L-aspatyl-L-glutamyl-L-valyl-L aspartic acid amide) (Z-DEVD-R110). After cell lysis, using a buffer that rapidly permeabilises cells, the active caspase-3 and caspase-7 can cleave the profluorescent substrate. The Z-DEVD-R110 peptide sequence that is cleaved to elicit a fluorescence signal is recognised by both caspase-3 and caspase-7. Following excitation at 485nm the rhodamine 110 leaving group becomes intensely fluorescent, and fluorescence emission is measured using a fluorescence plate-reader. The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample. The kit was used according to manufacturer's instructions with minor alterations. The ratio of substrate to buffer was maintained at 1:100 but volumes of sample per well in 96-well plate and Apo-ONE mixed reagent were reduced from 100µl to 50µl but the ratio of media:reagent was maintained at 1:1 as recommended.

The caspase-3/7 assay does not discriminate between the two caspases, therefore the affinity of the substrate for caspases-3 and –7 was investigated. A standard curve using purified recombinant caspase-3 and caspase-7 and produced a linear increase in fluorescence linear over a wide range of RFU (r^2= 0.9805 and 0.9975 for caspases-3 and-7 respectively).

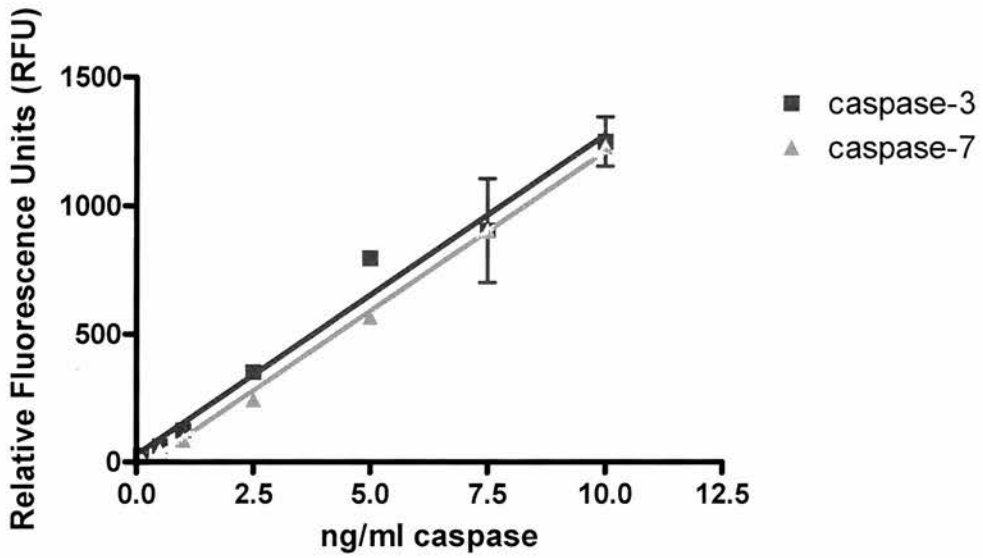


Figure 6.7: Caspase-3 and Caspase-7 standard curves

A dilution series of purified, active, recombinant, human caspases-3 and –7 (0.1ng/ml to 10ng/ml) was prepared in DMEM media in duplicate in a white-walled 96-well plate. Fluorescence measurements were made 21hours after addition of the substrate. Results are expressed as mean and S.D. of duplicates.

6.3.6.3 Data analysis

At the 24-hour timepoint selected, in addition to the ADP:ATP assay and caspase-3/7 assay, an SRB assay was carried out. By normalising the data from ADP:ATP and caspase assays to the OD from the SRB assay (which is relative to the number of living cells there) the cells that have already died as a consequence of drug treatment are accounted for, and only indication of cell death in the cells that are still alive is determined. Without normalisation of ATP data to SRB values to account for a reduction in the number of living cells, the ATP assay could be used as another cytotoxicity assay to determine cell death.

6.4 METHODS: RNA EXTRACTION AND qRT-PCR

6.4.1 RNA Extraction

Cultured cells (cell lines and hepatocytes for RNA analysis at start of drug treatment) were approximately 70% confluent in T75 tissue culture flasks. Fresh hepatocytes (for RNA analysis when they arrived from UKHTB) were centrifuged for 5minutes at 70g and supernatant discarded. Total RNA was then prepared from cells using Tri reagent, as per manufacturer's instructions, using 7.5mls Tri reagent per $75cm^2$ tissue culture flask or per hepatocyte cell pellet. Samples in Tri reagent were frozen at -70°C until processing, which occurred within 1 month.

Frozen samples in tri reagent were removed from -70°C and thawed in a cold-water bath, before addition of 0.2ml chloroform per ml of Tri reagent used initially. Samples were shaken vigorously, allowed to stand at RT for 15mins and then spun at 12000g for 15mins at 4°C in SS34 rotor in Sorvall® RC-6 centrifuge. The aqueous phase was transferred to a fresh tube and 0.5ml isopropanol added per ml of tri reagent used initially. Samples were allowed to stand for 10mins at RT before another spin at 12000g at 4°C for 10mins during which RNA precipitate formed a pellet at the bottom of the tube. The interphase and organic phase were stored at this stage at 4°C to allow subsequent isolation of protein. The supernatant was removed from tube and pellet of RNA was washed with 1ml of 75% ethanol per 1ml Tri reagent used in sample preparation. Samples were vortexed, and re-spun at 12000g at 4°C for 10mins. Most of the supernatant was removed and pellet was resuspended in 75% ethanol solution and stored at -20°C for up to 3 months. RNA samples were later removed from -20°C freezer and centrifuged at 16000g for 10mins at 4°C. Supernatant was removed and the pellet was air-dried for 15mins, then dissolved in 50µl distilled RNase-free water and heated at 55°C for 10mins in a thermal cycler to unfold tightly packed RNA.

6.4.2 DNase Treatment of RNA Samples

RNA samples were either treated with DNaseI and an RNase inhibitor (H1a to H22d, Ht1 to Ht31, and immortalised hepatocytes), or TURBO DNA-free™ (samples Ht32 to Ht49, H23a to H30d and hepatoma cell lines) to ensure that any contaminating

DNA was eliminated and to protect RNA samples from degradation. RNA concentration was determined by spectrophotometry on a NanoDrop, and the RNA was stored at -70°C until required for qRT-PCR.

6.4.3 Quantification of RNA by NanoDrop

1µl of the stock RNA sample was loaded onto NanoDrop Spectrophotometer and the NanoDrop Software determined RNA concentrations and O.D. 280 to 260 ratios.

6.4.4 Assessment of RNA Integrity

Many of the RNA samples analysed in these studies are from human tissue, and therefore an accurate and reproducible measurement of the quality of the RNA samples to be used for qRT-PCR analysis was required. The RNA 6000 Nano Assay was used to determine the quality of extracted RNA. 1µl of each RNA sample to be analysed was pipetted into one well on a RNA 6000 Nano chip, and following manufacturer's instructions the chip was loaded and run on an Agilent 2100 Bioanalyser. The samples are separated by electrophoresis and following detection of fluorescence the Bioanalyser software generates an electrophoretic trace and gel-like image (Figure 6.8).

Visual interpretation of the images produced by the Bioanalyser software is user-dependent. Therefore, to standardise determination of RNA quality, RNA integrity numbers (RIN) can be calculated by an Agilent software tool. More than 1300 RNA samples of varying qualities were used to develop the software. The entire electrophoretic trace is taken into account, and RIN numbers are assigned ranging from 1 (most degraded) to 10 (most in intact), which gives an indication of the quality of the RNA. The determination of RIN by the Agilent Bioanalyser is reported to be one of the most reliable and reproducible ways in which to determine RNA quality (Imbeaud et al, 2005). RIN numbers of the RNA samples from fresh human hepatocytes are shown in table 6.6. Sample H24d (from donor 10) was excluded from qRT-PCR analysis of gene expression, as RIN of 2.5 was too low. Excluding this sample, RNA from fresh hepatocytes used for qRT-PCR had RINs

ranging from 6.1 to 9.4, which was found to be of suitable quality for this application.

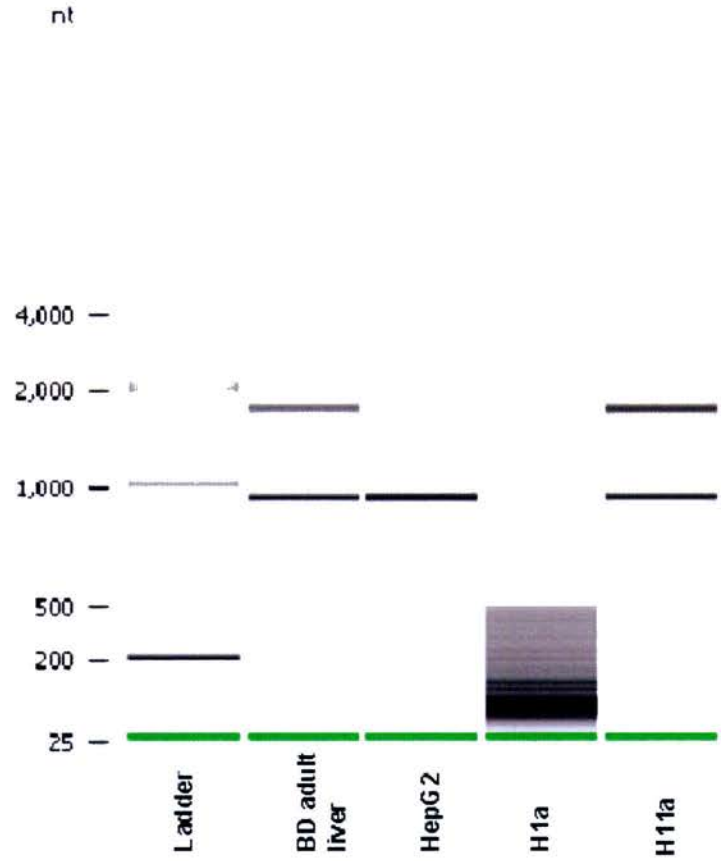


Figure 6.8: Agilent Assessment of RNA integrity

RNA ladder and RNA samples were loaded onto a RNA 6000 Nano chip and run on an Agilent 2100 Bioanalyser according to manufacturer's instructions. A representative gel-like image is shown with samples of human adult liver (BD Biosciences, used as qRT-PCR standard), HepG2, H1a and H11a RNA are shown.

Table 6.6: RNA integrity numbers (RIN) obtained for RNA samples, using an Agilent 2100 Bioanalyser and Agilent software

Hepatocyte Donor	Sample Code	RIN
1	H3a	8.4
	H2d	8.7
2	H5a	7.8
	H6d	8.9
3	H9a	8.0
	H10d	7.1
4	H11a	6.7
	H12d	6.7
5	H13a	6.7
	H14d	7.5
6	H15a	6.7
	H16d	7.8
7	H17a	8.3
	H18d	8.9
8	H19a	9.1
	H20d	9.3
9	H21a	7.9
	H22d	8.6
10	H23a	8.3
	H24d	2.5
11	H25a	8.1
	H26d	8.0
12	H27a	7.1
	H28d	7.6
13	H29a	7.6
	H30d	7.9
T1	Ht1	8.5
	Ht2	7.6
	Ht3	8.7
	Ht4	8.7
T2	Ht5	9.4
	Ht6	8.8
	Ht7	8.8
	Ht8	8.0
T3	Ht9	9.0
	Ht10	8.3
	Ht11	9.0
T4	Ht13	8.2
	Ht14	7.8
	Ht15	8.3
T5	Ht17	7.4
	Ht18	7.1

Hepatocyte Donor	Sample Code	RIN
	Ht19	7.9
	Ht20	6.4
T6	Ht21	7.5
	Ht22	7.0
	Ht23	8.0
	Ht24	8.2
T7	Ht25	6.7
	Ht26	7.2
	Ht27	7.3
T8	Ht29	6.7
	Ht30	6.1
	Ht31	7.4
	Ht32	8.0
T9	Ht33	8.6
	Ht34	8.9
	Ht35	8.8
	Ht36	8.4
T10	Ht39	8.8
	Ht40	8.2
	Ht41	8.6
	Ht42	8.5
T11	Ht45	8.5
	Ht46	7.4
	Ht47	7.2
	Ht48	7.6

6.4.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed using a real-time cycler Rotor-gene 3000 and QuantiTect®SYBR® Green RT-PCR Kit, according to manufacturer's instructions. Primers for each gene (see table 6.7) were designed using Primer3 software (Whitehead Institute for Biomedical Research, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and produced by either CRUK Oligo Synthesis Lab or Sigma Genosys. For primer sequences and NCBI accession numbers and product sizes (bp) of primers used for RT-PCR please refer to table 6.7. A standard curve was generated using known concentrations (dependent upon gene to be investigated) of human liver total RNA (BD Biosciences). Reactions were run in triplicate along with two additional reactions: one in which reverse transcriptase was omitted to allow assessment of genomic DNA contamination in each RNA sample, and the other containing no template (water as a substitute) to assess contamination of reagents during set-up of the reaction and primer dimer. Quantification of all genes is relative to the geometric mean of two reference genes. Samples were heated for 30minutes at 50°C to allow reverse transcription, followed by PCR initial activation step for 15minutes at 95°C. Following this, 40 cycles of a 3-step cycling process was performed: denaturation for 15 seconds at 94°C, annealing for 30 seconds at 57°C, then extension for 30 seconds at 72°C. All PCR products were checked by melt curve analysis of the PCR product, by stepwise increase in temperature by 1°C every 5 seconds from 65-99°C in the final step.

Table 6.7: Primer sequences for qRT-PCR

Where F=forward and R=reverse

GENE NAME	GENE ABBREVIATION	GenBank ACCESSION NUMBER	FORWARD (F) and REVERSE (R) PRIMER SEQUENCE (5' to 3')	Product Size (bp)
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	NM_000499	F: CTTGGACCTCTTTGGAGCTG R: CGAAGGAAGAGTGTCGGAAG	212
Cytochrome P450, family 3, subfamily A, polypeptide 4	CYP3A4	NM_017460	F: GAAACACAGATCCCCCTGAA R: TCTGGTGTTCTCAGGCACAG	162
Albumin	ALB	NM_000477	F: TGGCACAATGAAGTGGGTAA R: CTGAGCAAAGGCAATCAACA	166
α -1-antitrypsin	AAT	NM_000295	F: ACCTGGGACAGTGAATCGAC R: ATAGGCTGAAGGCGAACTCA	205
Transthyretin	TTR	NM_000371	F: ATGGCTTCTCATCGTCTGCT R: TGTCATCAGCAGCCTTTCTG	178
α-foetoprotein	AFP	NM_001134	F: AGCTTGGTGGTGGATGAAAC R: CCCTCTTCAGCAAAGCAGAC	248
UDP glucuronosyltransferase 1 family, polypeptide A*	UGT1A*	>1	F: TGTTCTGGGTGGAGTTTGGTG R: AGGAAACCAATCACGTCCAA	-
UDP glucuronosyltransferase 1 family, polypeptide B*	UGT2B*	>1	F: TGAGACAATGGCAAAAGCTG R: ACATTGGCCCTTTCTTCTGA	-
ATP-binding cassette, subfamily B (MDR), member 1	ABCB1	NM_000927	F: AGGCCAACATACATGCCTTC R: CCTTCTCTGGCTTTGTCCAG	220
Pregnane X receptor	PXR	NM_033013	F: TGTTCAAAGGCATCATCAGC R: CTGCAGTGTCTTCCAGCAG	189
Hypoxia-inducible factor 1α	HIF-1α	NM_001530	F: GAAAGCGCAAGTCCTCAAAG R: TGGGTAGGAGATGGAGATGC	167

GENE NAME	GENE ABBREVIATION	GenBank ACCESSION NUMBER	FORWARD (F) and REVERSE (R) PRIMER SEQUENCE (5' to 3')	Product Size (bp)
Heatshock protein 70kDA	HSP-70	NM_021979	F: GCACCGGTAAGGAAAACAAA R: TTTCTCGTCTTCCACCGTCT	191
Cyclin-dependent kinase inhibitor 1A (p21)	p21	NM_000389	F: GAGCGATGGAACTTCGACTT R: CAGGTCCACATGGTCTTCCT	201
18S ribosomal RNA	18S	X03205	F: AAACGGCTACCACATCCAAG R: CCTCCAATGGATCCTCGTTA	155
β2-microglobulin	B2M	NM_004048	F: CTCACGTCATCCAGCAGAGA R: TCTTTTTCAGTGGGGGTGAA	198
Glyceraldehyde-3-phosphate-dehydrogenase	GAPDH	NM_002046	F: CGACCACTTTGTCAAGCTCA R: AGGGGAGATTCAGTGTGGTG	203

6.4.6 Size of qRT-PCR Products

The size of the products amplified by qRT-PCR (see table 6.7) was verified by UV visualisation on 2%-agarose gels after ethidium bromide was added in the sample-loading buffer. A representative gel is shown in figure 6.9.

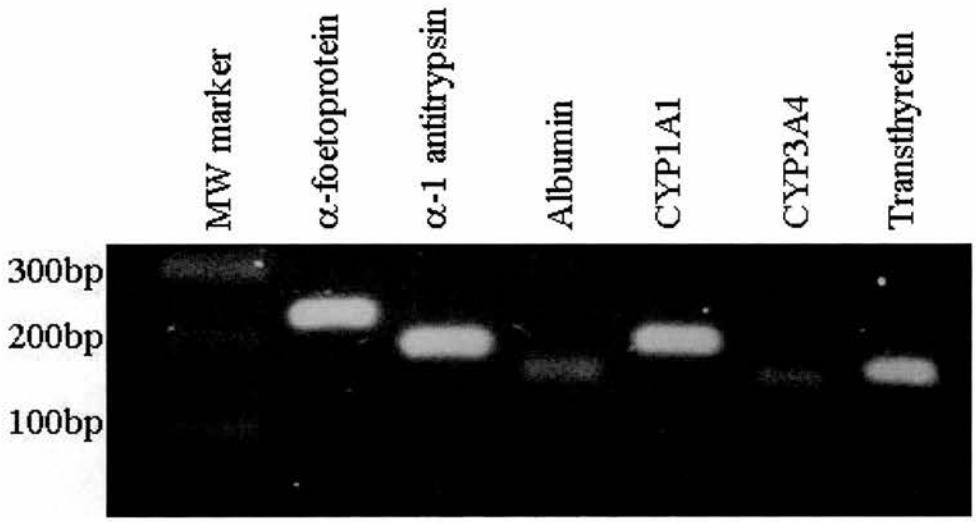


Figure 6.9: Verification of qRT-PCR product sizes

Samples amplified by qRT-PCR were loaded onto a 2% agarose and product sizes were visualised under UV light.

6.4.7 Analysis of qRT-PCR Data

Each sample was run in triplicate, and unknowns calculated from the standard curve. The mean of triplicates from the gene of interest was normalised to the geometric mean of triplicates of two reference genes. To evaluate differences in expression between the different cell types, statistical analysis was performed using a non-parametric Freidman test.

6.5 METHODS: PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

The technique of protein extraction and Western Blotting was attempted for analysis of the same proteins for which mRNA levels were measured. Due to the limited number of hepatocytes available, it was not possible to obtain enough cells specifically for protein analysis. Protein was therefore extracted from Tri reagent from these samples however there was little yield and the protein samples generated in this way were unfortunately unsuitable for Western blot analysis.

6.5.1 Protein Isolation from Tri Reagent

Phenol-ethanol supernatants, saved from RNA extraction of the same samples using tri reagent, were taken out of storage at 4°C. Protein was precipitated from this solution with 1.5ml isopropanol per ml of Tri reagent used in the initial sample preparation. Samples were allowed to stand for 10mins at room temperature, and then centrifuged at 12000g for 10 mins at 4°C. The supernatant was then discarded and the pellet washed with 0.3M guanidine hydrochloride/95% ethanol, using 2ml per 1ml Tri reagent used in sample preparation. The sample was then incubated in wash solution for 20 mins at room temperature, before centrifugation at 7500g for 5mins at 4°C. This washing step was repeated 3 times in total. After removal of the supernatant, 2ml of 100% ethanol was added to the protein pellet, vortexed, and allowed to stand for 20mins at room temperature. Samples were then centrifuged at 7500g for 5mins at 4°C, and pellets dried using a SpeedyVac for 15mins. The protein pellet was then dissolved in 1% SDS by repeated pipetting up and down. Samples were then centrifuged at 10000g for 10mins at 4°C, to remove any insoluble material. The supernatant was then transferred to a new tube, and stored at –20°C.

6.5.2 Bicinchoninic Acid Protein (BCA) Assay

To determine protein concentration a BCA assay was performed according to manufacturer's instructions. A concentration range of bovine serum albumin was used to generate a standard curve. Absorbance of the standard curve and protein lysates was then measured at 570nm on a microplate reader, allowing determination of unknown protein concentration.

6.5.3 Western Blotting

20μg of total cellular protein was separated by SDS-PAGE on a mini-gel apparatus and transferred to polyvinylidene fluoride membrane. Aliquots containing equal concentrations of protein were prepared, and their volumes equalised using lysis buffer. After addition of loading buffer to samples, they were denatured at 95°C for 5 mins in a heat block. Samples were loaded onto a 10-15% polyacrylamide gel (determined by MW of protein of interest), along with RPN-800 MW marker in at least one lane, and run at 80V for 15mins, then at 200V for 45mins-1hr. After electrophoresis, proteins were transferred to a permeabilised (with methanol) Immobilon-P membrane in a mini wet-transfer tank at 100V for 1hr. Transfer of protein to membrane was confirmed by staining of membranes with Ponceau S (0.1 %(w/v) in 5% acetic acid (v/v)).

Membranes were blocked using 5%-marvel in tris-buffered saline (TBS) for 1 hour at room temperature, and then incubated overnight with primary antibody in 2.5% marvel-TBS solution. For details of primary antibodies and suppliers please refer to table 6.8. Membranes were then washed 3 times for 5 minutes in TBS-Tween 20 (0.1%), then twice for 5minutes with 5% marvel-TBS-Tween 20, before incubation with secondary antibody for 1hour in 2.5% marvel-TBS. For details of secondary antibodies and suppliers please refer to table 6.9. Membranes were then washed 5 times for 5 minutes with TBS-Tween 20 and once for 5 minutes with TBS. After 1minute incubation with luminescence substrate solution, light emission was detected on photographic film in a dark room.

Table 6.8: Primary antibodies for Western Blot detection

PRIMARY ANTIBODY	DILUTION	RAISED IN	TYPE	SUPPLIER DETAILS
CYP1A1	1:1000	Sheep	HRP-conjugated	Chemicon International, AB1259
CYP3A4	1:2000	Rabbit	HRP-conjugated	Abcam, ab3572
Alpha-1 antitrypsin	1:1000	Chicken	HRP-conjugated	Chemicon International, AB3535
Albumin	1:5000	Mouse	HRP-conjugated	Abcam, ab24438
Alpha fetoprotein	1:5000	Rabbit	HRP-conjugated	Abcam, ab8201
Transthyretin	1:5000	Sheep	HRP-conjugated	Abcam, ab9015
Actin	1:120000	Mouse	HRP-conjugated	Oncogene

Table 6.9: Secondary antibodies for Western Blot detection

SECONDARY ANTIBODY	DILUTION	RAISED IN	TYPE	SUPPLIER DETAILS
Anti-rabbit IgG	1:5000	Goat	HRP-conjugated	Santa Cruz, sc-2004
Anti-mouse IgG	1:5000	Goat	HRP-conjugated	Santa Cruz, sc-2055
Anti-chicken IgY	1:5000	Goat	H&L chain specific, HRP-conjugated	Abcam, ab7118
Anti-sheep IgG	1:5000	Rabbit	H&L chain specific, HRP-conjugated	Calbiochem, #402100
Anti-mouse IgM (for actin)	1:8000	Goat	H& L chain specific, HRP-conjugated	Calbiochem #401225

CHAPTER 7: References

Abrahamse, S.L., van Runnard Heimel, P., Hartman, R.J., Chamuleau, R.A., van Gulik, T.M. (2003) Induction of necrosis and DNA fragmentation during hypothermic preservation of hepatocytes in UW, HTK, and Celsior solutions. Cell Transplantation 12(1):59-68.

Adamson, P.C., Zimm, S., Ragab, A.H., Steinberg, S.M., Balis, F., Kamen, B.A., Vietti, T.J., Gillespie, A., Poplack, D.G. (1990) A phase II trial of continuous-infusion 6-mercaptopurine for childhood solid tumors. Cancer Chemotherapy and Pharmacology 26(5)343-344. Abstract.

Adamson, P.C., Zimm, S., Ragab, A.H., Balis, F., Steinberg, S.M., Kamen, B.A., Vietti, T.J., Gillespie, A., Poplack, D.G. (1992) A phase II trial of continuous-infusion 6-mercaptopurine for childhood leukemia. Cancer Chemotherapy and Pharmacology 30(2)155-157.

Adamson, P.C., Widemann, B.C., Reaman, G.H., Seibel, N.L., Murphy, R.F., Gillespie, A.F., Balis, F.M. (2001) A phase I trial and pharmacokinetic study of 9-*cis*-retinois acid (ALRT1057) in pediatric patients with refractory cancer: a joint pediatric oncology branch, national cancer institute, and children's cancer group study. Clinical Cancer Research 7:3034-3039.

Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I., Knowles, BB. (1979) Controlled synthesis of HbsAg in a differentiated human liver carcinoma-derived cell line. Nature 282(5739):616-616. Abstract.

Aird, R.E., Cummings, J., Ritchie, A.A., Muir, M., Morris, R.E., Chen, H., Sadler, P.J., Jodrell, D.I. (2002) In vitro and in vivo activity and cross resistance profiles of novel ruthenium (II) organometallic arene complexes in human ovarian cancer. British Journal of Cancer 86(10):1652-1657.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. Molecular Biology of the Cell, 2002, 4th Edition, 1277-1278.

Alexander, J.J., Bey, E.M., Geddes, E.W., Lecatsas, G. (1976) Establishment of a continuously growing cell line from primary carcinoma of the liver. South African Medical Journal 50(54):2124-2128. Abstract.

Alexandre, E., Cahn, M., Abadie-Viollon, C., Meyer, N., Heyd, B., Mantion, G., Cinqualbre, J., David, P., Jaeck, D., Richert, L. (2002) Influence of pre-, intra- and post-operative parameters of donor liver on the outcome of isolated human hepatocytes. Cell Tissue Bank 3(4):223-233. Abstract.

Alley, M.C. Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., Boyd, M.R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Research 48(3):589-601.

Alvaro, D., Bendetti, A., Marucci, L., Delle Monache, M., Monterubbianesi, R., Di Cosimo, E., Perego, L., Macarri, G., Glaser, S., Le Sage, G., Alpini, G. (2000) The function of alkaline phosphatase in the liver: regulation of intrahepatic biliary epithelium secretory activities in the rat. Hepatology 32(2): 174-184.

Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., PAstan, I., Gottesman, M.M. (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annual Review of Pharmacology and Toxicology 39:361-398.

Anderson, K., Yin, L., MacDonald, C., Grant, M.H. (1996) Immortalized hepatocytes as in vitro model systems for toxicity testing: the comparative toxicity of menadione in immortilized cells, primary cultures of hepatocytes and HTC hepatoma cells. Toxicology in Vitro 10:721-727.

Andreasen, P.B. (1978) Hepatotoxicity in patients with liver disease. Archives of Toxicology Supplement 1:131-136. Abstract.

Aota, K., Azuma, M., Yamashita, T., Tamatani, T., Motegi, K., Ishimaru, N., Hayashi, Y., Sato, M. (2000) 5-Fluorouracil induces apoptosis through the suppression of NF-κB activity in human salivary gland cells. Biochemical and Biophysical Research Communications 273:1168-1174.

Arango, D., Wilson, A.J., Shi, Q., Corner, G.A., Aranes, M.J., Nicholas, C., Lesser, M., Mariadason, J.M., Augenlicht, L.H. (2004) Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. British Journal of Cancer 91:1931-1946.

Arotcarena, R., Cales, V., Berthelemy, P., Parent, Y., Malet, M., Etcharry, F., Ferrari, S., Pariente. (2006) Severe sinusoidal lesions: a serious and overlooked complication of oxaliplatin-containing chemotherapy? Gastroenterologie clinique et biologique 30(11):1313-1316.

Asbury, R.F., Rosenthal, S.N., Descalzi, M.E., Ratcliffe, R.L., Arseneau, J.C. (1980) Hepatic veno-occlusive disease due to DTIC. Cancer 45(10):2670-2674. Abstract.

Ashkenazi, A., Dixit, V.M. (1999) Apoptosis control by death and decoy receptors. Current Opinion in Cell Biology 11:255-260.

Aubrey, D.A. (1970) Massive hepatic necrosis after cyclophosphamide. British Medical Journal 3:588.

Aviles, A., Herrera, J., Ramos, E., Ambriz, R., Aguirre, J., Pizzuto, J. (1984) Hepatic injury during doxorubicin therapy. Archives of Pathology and Laboratory Medicine 108(11):912-913. Abstract.

Backus, H.H.J., Wouters, D., Ferreira, C.G., van Houten, V.M.M., Brakenhoff, R.H., Pinedo, H.M., Peters, G.J. (2003) Thymidylate synthase inhibition triggers apoptosis

via caspases-8 and –9 in both wild-type and mutant p53 colon cancer cell lines. European Journal of Cancer 39:1310-1317.

Ballet, F. (1997) Hepatotoxicity in drug development: detection, significance and solutions. Journal of Hepatology 26(suppl. 2):26-36.

Banasiak, D., Barnetson, A.R., Odell, R.A., Mameghan, H., Russel, P.J. (1999) Comparison between the clonogenic, MTT, and SRB assays for determining radiosensitivity in a panel of human bladder cancer cell lines and a ureteral cell line. Radiation Oncology Investigations 7:77-85.

Banerji, U., O'Donnell, A., Scurr, M., Pacey, S., Stapleton, S., Asad, Y., Simmons, L., Maloney, A., Raynaud, F., Campbell, M., Walton, M., Lakhani, S., Kaye, S., Workman, P., Judson, I. (2005) Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. Journal of Clinical Oncology 23(18):4152-4161.

Baughman, T.M., Graham, R.A., Wells-Knecht, K., Silver, I.S., Tyler, L.O., Wells-Knecht, M., Zhao, Z. (2005) Metabolic activation of pioglitazone identified from rat and human liver microsomes and freshly isolated hepatocytes. Drug Metabolism and Disposition 33(6): 733-738.

Bayliss, M.K; Skett, P. Isolation and culture of human hepatocytes. Human cell culture protocols, Methods in molecular medicine. 1996 Edited Jones G.E. 369-389. Published Humana Press 369-389.

Belt, R.J., McGregor, D., Haas, C.D., Bhatia, P.S. (1980) Cholestatic jaundice associated with chlorozotocin. Cancer Treatment Reports 64(12):1235-1239.

Berkovitch, M., Matsui, D., Zipursky, A., Blanchette, V.S., Verjee, Z., Giesbrecht, E., Saunders, E.F., Evans, W.E., Koren, G. (1996) Hepatotoxicity of 6-mercaptopurine in childhood acute lymphocytic leukaemia: pharmacokinetic characteristics. Medical and Pediatric Oncology 26(2):85-89. Abstract.

Bhargava, P., Marshall, J.L., Dahut, W., Rizvi, N., Trocky, N., Williams, J.I., Hait, H., Song, S., Holroyd, K.J.,Hawkins, M.J. (2001) A phase I and pharmacokinetic study of squalamine, a novel antiangiogenic agent, in patients with advance cancers. Clinical Cancer Research 7:3912-3919.

Biagini, C.P., Boissel, E., Borde, F., Bender, V.E., Bouskila, M., Blazy, F., Nicaise, L., Mignot, A., Cassio, D., Chevalier, S. (2006) Investigation of the hepatotoxicity profile of chemical entities using Liverbeads® and WIF-B9 in vitro models. Toxicology in Vitro 20(6):1051-1059.

Binda, D., lasserre-Bigot, D., Bonet, A., Thomassin, M., Come, M.P., Guinchard, C., Bars, R., Jacqueson, A., Richert, L. (2003) Time course of cytochromes P450 decline during rat hepatocyte isolation and culture: effect of L-NAME. Toxicology in Vitro 17:59-67.

Blanchard, N., Alexandre, E., Abadie, C., Lave, T., Heyd, B., Mantion, G., Jaeck, D., Richert, L., Coassolo, P. (2005) Comparison of clearance predictions using primary cultures and suspensions of human hepatocytes. Xenobiotica 35(1):1-15.

Blaner, W.S., Bonifacio, M.J., Feldman, H.D., Piantedosi, R., Saraiva, M.J.M. (1991) Studies on the synthesis and secretion of transthyretin by the human hepatoma cell line HepG2. FEBS 278(1,2):193-196.

Blanquicett, C., Johnson, M.R., Heslin, M., Diasio, R.B. (2002) Housekeeping gene variability in normal and carcinomatous colorectal and liver tissues: Applications in pharmacogenomic gene expression studies. Analytical Biochemistry 303:209-214.

Blower, P., de Wit, R., Goodin, S., Aapro, M. (2005) Drug-drug interactions in oncology: why are they important and can they be minimized? Critical Reviews in Oncology/Hematology 55(2):117-142.

Borozan, I., Chen, L., Sun, J., Tannis, L-L., Guindi, M., Rotstein, O.D., Heathcote, J., Edwards, A.M., Grant, D., McGilvray, I.D. (2006) Gene expression profiling of acute liver stress during living donor liver transplantation. American Journal of Transplantation 6:806-824.

Borst, P., Evers, R., Kool, M., Wijnholds, J. (2000) A family of drug transporters: the multidrug resistance-associated proteins. Journal of the National Cancer Institute 92(16): 1295-1302.

Borzelleca, J.F. (2000) Paracelsus: Herald of modern toxicology. Toxicological Sciences 52:2-4.

Brabec, V., Novakova, O. (2006) DNA binding mode of ruthenium complexes and relationship to tumour cell toxicity. Drug Resistance Updates 9(3):111-122. Abstract.

Bradbury, D.A., Simmons, T.D., Slater, K.J., Crouch, S.P.M. (2000) Measurement of the ADP:ATP ration in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. Journal of Immunological Methods 240:79-92.

Bradham, C.A., Plumpe, J., Manns, M.P., Brenner, D.A., Trautwein, C. (1998) Mechanisms of hepatic toxicity I. TNF-induced liver injury. American Journal of Physiology 275:G387-G392.

Brandon, E.F., Meijerman, I., Klijn, J.S., den Arend, D., Sparidans, R.W., Lazaro, L.L., Beijnen, J.H., Schellens, J.H. (2005) In-vitro cytotoxicity of ET-743 (Trabectidin, Yondelis), a marine anti-cancer drug, in the Hep G2 cell line: influence of cytochrome P450 and phase II inhibition, and cytochrome P450 induction. Anticancer Drugs 16(9):935-943. Abstract.

Brattain, M.G., Fine, W.D., Khaled, M., Thompson, J., Brattain, D.E. (1981) Heterogeneity of malignant cells from a human colonic carcinoma. Cancer Research 41:1751-1756.

Bross, P.F., Beitz, J., chen, G., Chen, X.H., Duffy, E., Kieffer, L., Roy, S., Sridhara, R., Rahman, A., Williams, G., Pazdur, R. (2001) Approval Summary: Gemtuzamab ozogamicin in relapsed acute myeloid leukaemia. Clinical Cancer Research 7:1490-1496.

Burczynski, M.E., McMillan, M., Ciervo, J., Li, L., Parker, J.B., Dunn II, R.T., Hicken, S., Farr, S., Johnson, M.D. (2000) Toxicogenomics-based discrimination of toxic mechanism in HepG2 human hepatoma cells. Toxicological Sciences 58:399-415.

Buss, N., Cammack, N. (2001) Measuring the effectiveness of antiretroviral agents. Antiviral Therapy 6(1):1-7.

Bustin, S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerise chain reaction assays. Journal of Molecular Endocrinology 25:169-193.

Bustin, S.A., Nolan, T. (2004) Pitfalls of quantitative real time reverse-transcription polymerase chain reaction. Journal of Biomolecular Techniques 15(3): 155-166.

Butterworth, B.E., Smith-Oliver, T., Earle, l., Loury, D.J., White, R.D., Doolittle, D.J., Working, P.K., Cattley, R.C., Jirtle, R., Michalopoulos, G. (1989) Use of primary cultures of human hepatocytes in toxicology studies. Cancer Research 49(5):1075-1084. Abstract.

Cai, J., Zhao, Y., Liu, Y., Ye, F., Song, Z., Qin, H., Meng, S., Chen, Y., Zhou, R., Song, X., Guo, Y., Ding, M., Deng, H. (2007) Directed differentiation of human embryonic stem cells into functional hepatic cells. Hepatology 45(5):1229-1239.

Canal, P., Gay, C., Dezeuze, A., Douillard, J.Y., Bugat, R., Brunet, R., Adenis, A., Herait, P., Lokiec, F., Mathieu-Boue, A. (1996) Pharmacokinetics and pharmacodynamics of irinotecan during a phase II clinical trial in colorectal cancer. Journal of Clinical Oncology 14:2688-2695.

Carlini, P., Papaldo, P., Fabi, A., Felici, A., Ruggeri, E.M., Milella, M., Ciccarese, M., Nuzzo, C., Cognetti, F., Ferretti, G. (2006) Liver toxicity after treatment with gefitinib and anastrozole: drug-drug interactions through cytochrome p450? Journal of Clinical Oncology 24(35):60-61.

Cavallari, A., Cillo, U., Nardo, B., Filipponi, F., Gringeri, E., Montalti, R., Vistoli, F., D'Amico, F., Faenza, A., Mosca, F., Vitale, A., D'Amico, D. (2003) A multicenter pilot prospective study comparing celsior and university of Wisconsin preserving solutions for use in liver transplantation. Liver Transplantation 9(8):814-821.

Ceci, G., Bella, M., Melissari, M., Gabrielli, M., Bocchi, P., Cocconi, G. (1988) Fatal hepatic vascular toxicity of DTIC. Is it really a rare event? Cancer 61(10):1988-1991. Abstract.

Chandra, P., Brouwer, K.L. (2004) The complexities of hepatic drug transport: current knowledge and emerging concepts. Pharmaceutical Research 21(5):719-735.

Chen, H-L., Wu, H-L., Fon, C-C., Chen, P-J., Lai, M-Y., Chen, D-S. (1998) Long-term culture of hepatocytes from human adults. Journal of Biomedical Science 5:435-440.

Clark, D.L., Andrews, P.A., Smith, D.D., DeGeorge, J.J., Justice, R.L., Beitz, J.G. (1999) Predictive value of preclinical toxicology studies for platinum anticancer drugs. Clinical Cancer Research 5:1161-1167.

Coeman, D.C., Verbeken, E.K., Nackaerts, K.L., Demedts, M.G., Vansteenkiste, J.F. (2000) A fatal case of cholestatic liver failure probably related to gemcitabine. Annals of Oncology 11:1503-1504.

Cohen, A.D., Luger, S.M., Sickles, C., Mangan, P.A., Porter, D.L., Schuster, S.J., Tsai, D.E., Nasta, S. (2002) Gemtuzumab ozogamicin (Mylotarg) monotherapy for relapsed AML after hematopoietic stem cell transplant: efficacy and incidence of hepatic veno-occlusive disease. Bone Marrow Transplantation 30:23-28.

Colombo, P., Gunnarsson, K., Iatropoulos, M., Brughera, M. (2001) Toxicological testing of cytotoxic drugs (review). International Journal of Oncology 19:1021-1028.

Crary, G.S., Albrecht, J.H. (1998) Expression of cyclin-dependent kinase inhibitor p21 in human liver. Hepatology 28:738-743.

Crouch, S.P.M., Kozlowski, R., Slater, K.J., Fletcher, J. (1993) The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. Journal of Immunological Methods 160: 81-88. Abstract.

Cummins, J.M., Kohli, M., Rago, C., Kinzler, K.W., Vogelstein, B., Bunz, F. (2004) X-linked inhibitor of apoptosis protein (XIAP) is a nonredundant modulator of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. Cancer Research 64: 3006-3008.

Daemer, R.J., Feinstone, S.M., Alexander, J.J., Tully, J.G., London, W.T., Wong, D.C., Purcell, R.H. (1980) PLC/PRF/5 (Alexander) Hepatoma cell line: Further characterization and studies of infectivity. Infection and Immunity 30(2):607-611.

Dambach, D.M., Andrews, B.A., Moulin, F. (2005) New technologies and screening strategies for hepatotoxicity: Use of in vitro models. Toxicologic Pathology 33:17-26.

Davila, J.C., Rodriguez, R.J., Melchert, R.B., Acosta, D. (1998) Predictive value of in vitro model systems in toxicology. Annual Review of Pharmacology and Toxicology 38:63-96.

DeGeorge, J.J., Ahn, C-H., Andrews, P.A., Brower, M.E., Giorgio, D.W., Goheer, M.A., Lee-Ham, D.Y., McGuinn, W.D., Schmidt, W., Sun, C.J., Tripathi, S.C. (1997) Regulatory considerations for preclinical development of anticancer drugs. Cancer Chemotherapy and Pharmacology 41(3):173-185.

DeLeve, L.D., Wang, X.,, Kuhlenkamp, J.F., Kaplowitz, N. (1996) Toxicity of azathioprine and monocrotaline in murine sinusoidal endothelial cells and hepatocytes: The role of glutathione and relevance to hepatic veno occlusive disease. Hepatology 23(3):589-599.

DeLeve, L.D. (2003) Liver function and hepatotoxicity in cancer. Cancer Medicine 6. Accessed via National Center for Biotechnology Information (NCBI) Books website: http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=cmed.chapter.40122.

De Vita, V.T., Carbone, P.P., Owens, A.H. Jr., Gold, G.L., Krant, M.J., Edmonson, J. (1965) Clinical trials with 1,3-bis(2-chloroethyl)-1-nitrosurea, NSC-409962. Cancer Research 25(11):1876-1881.

Donald, S., Verscholyle, R.D., Edwards, R., Judah, D.J., Davies, R., Riley, J., Dinsdale, D., Lazaro, L.L., Smith, A.G., Gant, T.W., Greaves, P., Gescher, A.J. (2002) Hepatobiliary damage and changes in hepatic gene expression caused by the antitumor drug ecteinascidin-743 (ET-743) in the female rat. Cancer Research 62:4256-4262.

Donato, M.T., Serralta, A., Jimenez, N., Perez, G., Castell, J.V., Mir, J., Gomez-Lechon, M.J. (2005) Liver grafts preserved in celsior solution as a source of hepatocytes for drug metabolism studies: comparison with surgical liver biopsies. Drug Metabolism and Disposition 33(1):108-114.

Donehower, R.C., Karp, J.E., burke, P.J. (1986) Pharmacology and toxicity of high-dose cytarabine by 72-hour continuous infusion. Cancer treatment Reports 70(9):1059-1065.

Doria, M.I. Jr., Shepard, K.V., Levin, B., Riddell, R.H. (1986) liver pathology following hepatic arterial infusion chemotherapy. Hepatic toxicity with FUDR. Cancer 58(4):855-861. Abstract.

Doroshow, J.H., Synold, T.W., Gandara, D., Mani, S., Remick, S.C., Mulkerin, D., Hamilton, A., Sharma, S., Ramanathan, R.K., Lenz, H.J., Graham, M., Longmate, J., Takimoto, C.H., Ivy, P. (2003) Pharmacology of oxaliplatin in solid tumour patients with hepatic dysfunction: a preliminary report of the National Cancer Institute Organ Dysfunction Working Group. Seminars in Oncology (4Suppl15):14-19.

Dotto, G.P. (2000) p21(WAF1/Cip1): more than a break to the cell cycle? Biochimica et Biophysica Acta 1471(1): M43-56.

Dufour, D.R., Lott, J.A., Nolte, F.S., Gretch, D.R., Koff, R.S., Seeff, L.B. (2000) Diagnosis and monitoring of hepatic injury: I. performance characteristics of laboratory tests. Clinical Chemistry 46(12): 2027-2049.

Duverger, V., Murphy, A-M., Sheehan, D., England, K., Cotter, T.G., Hayes, I., Murphy, F.J. (2004) The anticancer drug mithramycin A sensitises tumour cells to apoptosis induced by tumour necrosis factor (TNF). British Journal of Cancer 90:2025-2031.

Eichelbaum, M., Kroemer, H.K., Mikus, G. (1992) Genetically determined differences in drug metabolism as a risk factor in drug toxicity. Toxicology Letters 64-65:115-122. Abstract.

Elefsiniotis, I.S., Pantazis, K.D., Ilias, A., Pallis, L., Mariolis, A., Glynou, I., Kada, H., Moulakakis, A. (2004) Tamoxifen induced hepatotoxicity in breast cancer patients with pre-existing liver steatosis: the role of glucose intolerance. European Journal of Gastroenterology and Hepatology 16(6):593-598. Abstract.

Eriguchi, M., Nonaka, Y., Yanagie, H., Yochizaki, I., Takeda, Y., Sekiguchi, M. (2003) A molecular biological study of anti-tumor mechanism of an anti-cancer agent Oxaliplatin against established human gastric cancer cell lines. Biomedicine and Pharmacotherapy 57:412-415.

Evans, W.E., Hon, Y.Y., Bomgaars, L., Coutre, S., Holdsworth, M., Janco, R., Kalwinsky, D., Keller, F., Khatib, Z., Margolin, J., Murray, J., Quinn, J., Ravindranath, Y., Ritchey, K., Roberts, W., Rogers, Z.R., Schiff, D., Steuber, C., Tucci, F., Komegay, N., Krynetski, E.Y., Relling, M.V. (2001) Preponderance of thiopurine S-methyltransferase deficiency and heterozygosity among patients intolerant to mercaptopurine or azothioprine. Journal of Clinical Oncology 19(8):2293-2301.

Farrell, G.C. (1994) Drug-induced liver disease. Churchill Livingstone. Pages 1-162 and 473-488.

Fattinger, K., Funk, C., Pantze, M., Weber, C., Reichen, J., Stieger, B., Meier, P.J. (2001) The endothelin antagonist Bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. Clinical Pharmacology and Therapeutics 69(4): 223-231.

Faubion, W.A., Gores, G.J. (1999) Death receptors in liver biology and pathobiology. Hepatology 29(1): 1-4.

Feenstra, J., Vermeer, R.J., Stricker, B.H.C.H. (1997) Fatal hepatic coma attributed to paclitaxel. Journal of the National Cancer Institute 89(8):582-583.

Feng, L., Zhao, N., Yao, X., Sun, X., Diao, X., Li, S., Li, Y. (2007) Histidine-tryptophan-ketoglutarate solution vs. University of Wisconsin solution for liver transplantation: a systematic review. Liver transplantation 13(8): 1125-1136.

Fernandez, F.G., Ritter, J., Goodwin, J.W., Linehan, D.C., Hawkins, W.G., Strasberg, S.M. (2005) Effect of steatohepatitis associated with irinotecan or oxaliplatin pre-treatment on resectability of hepatic colorectal metastases. Journal of the American College of Surgeons 200(6):848-853. Abstract.

Findlay, M.P.N., Raynaud, F., Cunningham, D., Iveson, A., Colins, D.J., Leach, M.O. (1996) Measurement of plasma 5-fluorouracil by high-performance liquid chromatography with comparison of results to tissue drug levels observed using in vivo 19F magnetic resonance spectroscopy in patients on a protracted venous infusion with or without interferon-alpha. Annals of Oncology 7:47-53.

Fink, S.L., Cookson, B.T. (2005) Apoptosis, pyroptosis and necrosis: mechanistic description of dead and dying eukaryotic cells. Infection and Immunity 73(4):1907-1916.

Fisher, B., Keenan, A.M., Garra, B.S., Steinberg, S.M., White, D.E., DiBisceglie, A.M., Hoofnagle, J.H., Yolles, P., Rosenberg, S.A., Lotze, M.T. (1989) Interleukin-2 induces profound reversible cholestasis: a detailed analysis in treated cancer patients. Journal of Clinical Oncology 7(12):1852-1862. Abstract.

Flowers, M.A., Heathcote, J., Wanless, I.R., Sherman, M., Reynolds, W.J., Cameron, R.G., Levy, G.A., Inman, R.D. (1990) Fulminant hepatitis as a consequence of reactivation of hepatitis infection after discontinuation of low-dose methotrexate therapy. Annals of Internal medicine 112(5):381-382. Abstract.

Floyd, J., Mirza, I., Sachs, B., Perry, M.C. (2006) Hepatotoxicity of chemotherapy. Seminars in Oncology 33(1)50-67.

Foitl, D.R., Hyman, G., Lefkowitch, J.H. (1989) Jaundice and intrahepatic cholestasis following high-dose megestrol acetate for breast cancer. Cancer 63(3):438-439. Abstract.

Fulda, S., Meyer, E., Friesen, C., Susin, S.A., Kroemer, G., Debatin, K-M. (2001) Cell type specific involvement of death receptor and mitochondrial pathways in drug-induced apoptosis. Oncogene 20:1063-1075.

Ganten, T.M., Koschny, R., Sykora, J., Schulze-Bergkamen, H., Buchler, P., Haas, T.L., Schader, M.B., Untergasser, A., Stremmel, W., Walczak, H. (2006) Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. Clinical Cancer Research 12(8):2640-2646.

Garcia, M., Rager, J., Wang, Q., Strab, R., Hidalgo, I.J., Owen, A., Li, J. (2003) Cryopreserved human hepatocytes as alternative in vitro model for cytochrome p450 induction studies. In Vitro Cell Dev Biol Anim. 39(7):283-287. Abstract.

Geick, A., Eichelbaum, M., Burk, O. (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. The Journal of Biological Chemistry 276(18): 14581-14587.

George, C.B., Mansour, R.P., Redmond, J.3rd., Gandara, D.R. (1984) Hepatic dysfunction and jaundice following high-dose cytosine arabinoside. Cancer 54(11):2360-2362.

Gibson, G., Skett, P. Introduction to Drug Metabolism (2001) 3rd Edition. Pages 37-70 and 87-159.

Goldberg, J.W., Lidsky, M.D. (1985) Cyclophosphamide associated hepatotoxicity. Southern Medical Journal 78:222-223. Abstract.

Gomez-Lechon, M.J., Ponsoda, X., O'Connor, E., Donao, T., Jover, R., Castell, J.V. (2003) Diclofenac induces apoptosis in hepatocytes. Toxicology in vitro 17:675-680.

Gomez-Lechon, M.J., Donato, M.T., Castell, J.V., Jover, R. (2003) Human hepatocytes as a tool for studying toxicity and drug metabolism. Current Drug Metabolism 4:292-312.

Gonzalez, F.J., Yu., A.M. (2006) Cytochrome P450 and xenobiotic receptor humanized mice. Annual review of pharmacology and toxicology 46:41-64.

Goss, P.E., Baptiste, J., Fernandes, B., Baker, M., Dennis, J.W. (1994) A phase I study of swainsonine in patients with advanced malignancies. Cancer Research 54(6):1450-1457.

Goss, P.E., Reid, C.L., Bailey, D., Dennis, J.W. (1997) Phase IB clinical trial of the oligosaccharide processing inhibitor swainsonine in patients with advanced malignancies. Clinical Cancer Research 3:1077-1086.

Graham, G., Muhsin, M., Kirkpatrick, P. (2004) Fresh from the pipeline: Oxaliplatin. Nature Reviews Drug Discovery 3:11-12.

Graham, M.A., Lockwood, G.F., Greenslade, D., Brienza, S., Bayssas, M., Gamelin, E. (2000) Clinical pharmacokinetics of Oxaliplatin: a critical review. Clinical Cancer Research 6:1205-1218.

Green, L., Donehower, R.C. (1984) Hepatic toxicity of low doses of mithramycin in hypercalcemia. Cancer Treatment Reports 68(11):1379-1381. Abstract.

Guengerich, F.P. (1999) Cytochrome P450 3A4: Regulation and role in drug metabolism. Annual Review of Pharmacology and Toxicology 39:1-17.

Guengerich, F.P., MacDonald, J.S. (2007) Applying mechanisms of chemical toxicity to predict drug safety. Chemical Research in Toxicology 20(3):344-369.

Guichard, S., Else, R., Reid, E., Zeitlin, B., Aird, R., Muir, M., Dodds, M., Fiebig, H., Sadler, P., Jodrell, D. (2006) Anti-tumour activity in non-small cell lung cancer models and toxicity profiles for novel ruthenium(II) based organo-metallic compounds. Biochemical Pharmacology 71(4):408-415.

Guichard, S.M., Macpherson, J.S., Mayer, I., Reid, E., Muir, M., Dodds, M., Alexander, S., Jodrell, D.I. (2007) Gene expression predicts differential capecitabine metabolism, impacting on both pharmacokinetics and antitumour activity. European Journal of Cancer doi:10.1016/j.ejca.2007.10.023.

Guillouzo, A. (2000) Liver cell models in in vitro toxicology. Environmental Health Perspectives 106Suppl2: 511-532.

Gunawan, B., Kaplowitz, N. (2004) Clinical perspectives on xenobiotic-induced hepatotoxicity. Drug Metabolism Reviews 36:301-312.

Handschin, C., Meyer, U.A. (2003) Induction of drug metabolism: the role of nuclear receptors. Pharmacological Reviews 55(4):649-673.

Hao, D., Hammond, L.A., Eckhardt, S.G., Patnaik, A., Takimoto, C.H., Schwartz, G.H., Goetz, A.D., Tolcher, A.W., McCreery, H.A., Mamun, K., Williams, J.I., Holroyd, K.J., Rowinsky, E.K. (2003) A phase I pharmacokinetic study of squalamine, an aminosterol angiogenesis inhibitor. Clinical Cancer Research 9:2465-2471.

Harmsen, S., Meijerman, I., Beijnen, J.H., Schellens, J.H. (2007) The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology. Cancer Treatment Reviews 33(4): 369-380.

Harries, H.M., Fletcher, S.T., Duggan, S.M., Baker, V.A. (2001) The use of genomics technology to investigate gene expression changes in cultured human liver cells. Toxicology in Vitro 15:399-405.

Harris, A.J., Dial, S.L., Casciano, D.A. (2004) Comparison of basal gene expression profiles and effects of hepatocarcinogens on gene expression in cultured primary human hepatocytes and HepG2 cells. Mutation Research 549:79-99.

Hartley, D.P., Dai, X., He, Y.D., Carlini, E.J., Wang, B., Huskey, S.E., Ulrich, R.G., Rushmore, T.H., Evers, R., Evans, D.C. (2004) Activators of the rat pregnane X receptor differentially modulate hepatic and intestinal gene expression. Molecular Pharmacology 65(5): 1159-1171.

Haskell, C.M., Canellos, G.P., Leventhal, B.G., Carbone, P.P., Block, J.B., Serpick, A.A., Selawry, O.S. (1969) L-asparaginase: therapeutic and toxic effects in patients with neoplastic disease. The New England Journal of Medicine 281(19):1028-1034. Abstract.

Hassen, W., El Golli, E., Baudrimont, I., Mobio, A.T., Ladjimi, M.M., Creppy, E.E., Bacha, H. (2005) Cytotoxicity and Hsp 70 induction in Hep G2 cells in response to zearalenone and cytoprotection by sub-lethal heat shock. Toxicology 207(2): 293-301.

Heddle, R., Calvert, A.F. (1980) Hydoxyurea induced hepatitis. The Medical Journal of Australia 1(3):121. Abstract.

Heffelfinger, S.C., Hawkins, H.H., Barrish, J., Taylor, L., Darlinton, G.J. (1992) SK HEP-1: A human cell line of endothelial origin. In Vitro Cellular and Developmental Biology 28A:136-142.

Hengstler, J.G., Ringel, M., Biefang, K., Hammel, S., Milbert, U., Gerl, M., Klebach, M., Diener, B., Platt, K.L., Bottger, T., Steinberg, P., Oesch, F. (2000) Cultures with cryopreserved hepatocytes: applicability for studies of enzyme induction. Chemico-Biological Interactions 125:51-73.

Hengstler, J.G., Utesch, D., Steinberg, P., Platt, K.L., Diener, B., Ringel, M., Swales, N., Fischer, T., Biefang, K., Gerl, M., Bottger, T., Oesch, F. (2000) Cryopreserved primary hepatocytes as a constantly available in vitro model for the evaluation of human and animal drug metabolism and enzyme induction. Drug metabolism Reviews 32(1):81-118.

Hewes, J.C., Riddy, D., Morris, R.W., Woodrooffe, A.J., Davidson, B.R., Fuller, B. (2006) A prospective study of isolated human hepatocyte function following liver resection for colorectal liver metastases: the effects of prior exposure to chemotherapy. Journal of Hepatology 45(2):263-270.

Hewitt, N.J., Buhring, K.U., Dasenbrock, J., Haunschild, J., Ladstetter, B., Utesch, D. (2001) Studies comparing in vivo:in vitro metabolism of three pharmaceutical compounds in rat, dog, monkey, and human using cryopreserved hepatocytes, microsomes, and collagen gel immobilized hepatocyte cultures. Drug Metabolism and Disposition 29(7):1042-1050.

Higashi, T., Isomoto, A., Tyuma, I., Kakishita, E., Uomoto, M., Nagai, K. (1985) Quantitative and continuous analysis of ATP release from blood platelets with firefly luciferase luminescence. Thrombosis and Haemostasis 53(1):65-69. Abstract.

Hinkel, A., Berges, R.R., Pannek, J., Schulze, H., Senge, T. (1996) Cyproterone acetate in the treatment of advanced prostatic cancer: retrospective analysis of liver toxicity in the long-term follow-up of 89 patients. European Urology 30(4):464-470. Abstract.

Holownia, A., Braszko, J.J. (2004) Tamoxifen cytotoxicity in hepatoblastoma cells stably transfected with human CYP3A4. Biochemical Pharmacology 67:1057-1064.

Holt, M.P., Ju, C. (2006) Mechanisms of drug-induced liver injury. The AAPS Journal 8(1):E48-54.

Hoofnagle, J.H., Dunsheiko, G.M., Schafer, D.F., Jones, E.A., Micetich, K.C., Young, R.C., Costa, J. (1982) Reactivation of chronic hepatitis B infection by cancer chemotherapy. Annals of Internal Medicine 96(4):447-449. Abstract.

Hoth, D., Woollet, P., Green, D., Macdonald, J., Schein, P. (1978) Phase I studies on chlorozotocin. Clinical Pharmacology and Therapeutics 23(6):712-722. Abstract.

Hruban, R.H., Sternberg, S.S., Meyers, P., Fleisher, M., Menendez-Botet, C., Boitnott, J.K. (1991) Fatal thrombocytopenia and liver failure associated with carboplatin therapy. Cancer Investigation 9(3):263-268. Abstract.

Huang, C.M., Elin, R.J., Ruddel, M., Sliva, C., Lotze, M.T., Rosenberg, S.A. (1990) Changes in laboratory results for cancer patients treated with interleukin-2. Clinical Chemistry 36(3):431-434. Abstract.

Hudes, G., Haas, N., Yeslow, G., Gillon, T., Gunnarsson, P.O., Ellman, M., Nordle, O., Eriksson, B., Miller, L., Cisar, L., Kopreski, M., Viaro, D., Hartley-Asp, B. (2002) Phase I clinical and pharmacologic trial of intravenous estramustine phosphate. Journal of Clinical Oncology 20(4):1115-1125.

Huggett, J., Dheda, K., Bustin, S., Zumla, A. (2005) Real-time RT-PCR normalization; strategies ad considerations. Genes and Immunity 6(4): 279-284.

Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E., Higgins, C.F. (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 346 (6282): 362-365.

Ilan, Y. (2002) Towards a bank of cryopreserved hepatocytes: which cells to freeze? (Editorial) Journal of Hepatology 37:145-146.

Imanishi, H., Okamura, N., Yagi, M., Noro, Y., Moriya, Y., Nakamura, T., Hayakawa, A., Takeshima, Y., Sakaeda, T., Matsuo, M., Okumura, K. (2007) Genetic polymorphisms associated with adverse events and elimination of methotrexate in childhood acute lymphoblastic leukaemia and malignant lymphoma. Journal of Human Genetics 52:166-171.

Imbeaud, S., Graudens, A., Boulanger, V., Barlet, X., Zaborski, P., Eveno, E., Mueller, O., Schroeder, A., Auffray, C. (2005) Towards standardization of RNA quality assessment using inter-independent classifiers of microcapillary electrophoresis traces. Nucleic Acids Research 33(5):e56.

Ingelman-Sundberg, M. (2001) Genetic Susceptibility to adverse effects of drugs and environmental toxicants: the role of the CYP family of enzymes. Mutation Research 482(1-2): 11-19.

Ishak, K.G., Zimmerman, H.J. (1995) Morphologic spectrum of drug-induced hepatic disease. Gastroenterology clinics of North America 24(4):759-786.

Iyer, L., King, C.D., Whitington, P.F., Green, M.D., Roy, S.K., Tephly, T.R., Coffman, B.L., Ratain, M.J. (1998) Genetic predisposition to metabolism of irinotecan (CPT-11): Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. The Journal of Clinical Investigation 101(4): 847-854.

Jaeschke, H., Gores, G.J., Cederbaum, A.I., Hinson, J.A., Pessayre, D., Lemasters, J.J. (2002) Mechanisms of hepatotoxicity. Toxicological Sciences 65:166-176.

Jaeschke, H. (2003) Are cultured liver cells the right tool to investigate mechanisms of liver disease or hepatotoxicity? Hepatology 38(4):1053-1055.

Jaeschke, H., Lemasters, J.J. (2003) Apoptosis versus oncotic necrosis in hepatic ischaemia/ reperfusion injury. Gastroenterology 125:1246-1257.

Jaeschke, H., Hasegawa, T. (2006) Role of neutrophils in acute inflammatory liver injury. Liver International 26(8):912-919.

Jeha, S., Gandhi, V., Chan, K.W., McDonald, L., Ramirez, I., Madden, R., Rytting, M., Brandt, M., Keating, M., Plunket, W., Kantarjian, H. (2004) Clofarabine, a novel nucleoside analog, is active in pediatric patients with advanced leukaemia. Blood 130(3):784-789.

Jia, Z., Zhang, J., Wei, D., Wang, L., Yuan, P., Le, X., Li, Q., Yao, J., Xie, K. (2007) Molecular basis of the synergistic antiangiogenic activity of bevacizumab and mithramycin A. Cancer Research 67(10):4878-4885.

Jiang, S., Song, M.J., Shin, E-C., Lee, M-O., Kim, S.J., Park, J.H. (1999) Apoptosis in human hepatoma cell lines by chemotherapeutic drugs via Fas-dependent and Fas-independent pathways. Hepatology 29(1):101-110.

Johnson, D.H., Greco, F.A., Wolff, S.N. (1983) Etoposide-induced hepatic injury: a potential complication of high-dose therapy. Cancer Treatment Reports 67(11):1023-1024. Abstract.

Johnston, D.E. (1999) Special considerations in interpreting liver function tests. American Family Physician 59(8):2223-2230.

Jones, B,E., Czaja, M.J. (1998) Mechanisms of hepatic toxicity III. Intracellular signalling in response to toxic liver injury. American Journal of Physiology 275:G874-G878.

Joo, M., Chi, J.G., Lee, Y. (2005) Expressions of HSP70 and HSP27 in hepatocellular carcinoma. Journal of Korean Medical Science 20:829-834.

Kangas, L., Gronroos, M., Nieminen, A.L. (1984) Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents in vitro. Medical Biology 62(6):338-343.

Kantarjian, H.M., Gandhi, V., Kozuch, P., Faderl, S., Giles, F., Cortes, J., O'Brien, S., Ibrahim, N., Khuri, F., Du, M., Rios, M.B., Jeha, S., McLaughlin, P., Plunkett, W., Keeating, M. (2003) Phase I clinical and pharmacology study of clofarabine in patients with solid and hematologic cancers. Journal of Clinical Oncology 21(6):1167-1173.

Kaplowitz, N. (2001) Drug-induced liver disorders: implications for drug development and regulation. Drug Safety 24(7): 483-490.

Kaplowitz, N. (2005) Idiosyncratic drug hepatotoxicity. Nature Reviews Drug Discovery 4:489-499.

Kerr, J.F.R., Wyllie, A.H., Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implication in tissue kinetics. British Journal of Cancer 26: 239-257. Abstract.

Kiang, J.G., Tsokos, G.C. (1998) Heat shock protein 70kDa: molecular biology, biochemistry, and physiology. Pharmacology and Therapeutics 80(2): 183-201.

Kikkawa, R., Yamamoto, T., Fukushima, T., Yamada, H., Horii, I. (2005) Investigation of a hepatotoxicity screening system in primary cell cultures -"what biomarkers would need to be addressed to estimate toxicity in conventional and new approaches?". The Journal of Toxicological Sciences 30(1):61-72.

Kim, N.K., Kin, T-Y., Shin, S-G., Park, Y.I., Lee, J.A., Cho, Y-B., Kim, K.H., Kim, D-K., Heo, D.S., Bang, Y-J. (2001) A phase I study of *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-1,3-dioxolane] platinum (II) in patients with advanced malignancies. Cancer 91(8):1549-1556.

Kim, R., Tanabe, K., Uchida, Y., Emi, M., Inoue, H., Toge, T. (2002) Current status of the molecular mechanisms of anticancer drug-induced apoptosis. Cancer Chemotherapy and Pharmacology 50:343-352.

King, C.D., Rios, G.R., Green, M.D., Tephly, T.R. (2000) UDP-glucuronosyltransferases. Current Drug Metabolism 1:143-161.

King, P.D., Perry, M.C. (2001) Hepatotoxicity of chemotherapy. The Oncologist 6:162-176.

Koler R.D., Forsgren A.L. (1958) Hepatotoxicity due to chlorambucil. Report of a case. The Journal of the American Medical Association 167:316-317.

Kon, K., Kin, J.S., Jaeschke, H., Lemasters, J.J. (2004) Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. Hepatology 40(5):1170-1179.

Konig, J., Nies, A.T., Cui, Y., Leier, I., Keppler, D. (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. Biochimica et Biophysica acta 1461(2): 377-394.

Kostova, I. (2006) Ruthenium complexes as anticancer agents. Current Medicinal Chemistry 13(9):1085-1107. Abstract.

Kostrubsky, V.E., Strom, S.C., Hanson, J., Urda, E., Rose, K., Burleigh, J., Zocharski, P., Cai, H., Sinclair, J., Sahi, J. (2003) Evaluation of hepatotoxic potential of drugs by inhibition of bile-acid transport in cultured primary human hepatocytes and intact rats. Toxicological Sciences 76:220-228.

Kostrubsky, S.E., Sinclair, J.F., Strom, S.C., Wood, S., Urda, E., Stolz, D.B., Wen, Y.H., Kulkarni, S., Mutlib, A. (2005) Phenobarbital and phenytoin increased acetaminophen hepatotoxicity due to inhibition of UDP-glucuronosyltransferases in cultured human hepatocytes. Toxicological Sciences 87(1): 146-155.

Kregel, K.C. (2002) Molecular biology of thermoregulation. Invited review: Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. Journal of Applied Physiology 92:2177-2186.

Kresse, M., Latta, M., Kunstle, G., Riehle, H.M., van Rooijen, N., Hentze, H., Tiegs, G., Biburger, M., Lucas, R., Wendel, A. (2005) Kupffer cell-expressed membrane-bound TNF mediates melphalan hepatotoxicity via activation of both TNF receptors. Journal of Immunology 175(6): 4076-4083.

Kudryavtseva, E.I., Engelhardt, N.V. (2003) Requirement of 3D extracellular network for maintenance of mature hepatocyte morphology and suppression of alpha-fetoprotein synthesis in vitro. Immunology Letters 90:25-31.

Kumashiro, R., Kubota, T., Koga, Y., Tanaka, M., Inada, C., Kusaba, N., Yoshida, H., Hisamochi, A., Ide, T., Tomita, Y., Masumoto, N., Tanikawa, K., Iga, T., Sata, M. (2003) Association of troglitazone-induced liver injury with mutation of the cytochrome P450 2C19 gene.

Kwon, Y.H., Jovanovic, A., Serfas, M.S., Tyner, A.L. (2003) The Cdk inhibitor p21 is required for necrosis, but it inhibits apoptosis following toxin-induced liver injury. Journal of Biological Chemistry 278:30348-30355.

Laidlaw, S.T., Reilly, J.T., Suvarna, S.K. (1995) Fatal hepatotoxicity associated with 6-mercaptopurine therapy. Postgraduate Medical Journal 71(840):639. Abstract.

Laskin, D.L., Gardner, C.R., Price, V.F., Jollow, D.J. (1995) Modulation of macrophage functioning abrogates the acute hepatotoxicity of acetaminophen. Hepatology 21(4):1045-1050.

Lawen, A. (2003) Apoptosis- An introduction. BioEssays 25:888-896.

Lazarus, H.M., Herzig, R.H, Graham-Pole, J., Wolff, S.N., Phillips, G.L., Strandjord, S., Hurd, D., Forman, W., Gordon, E.M., Coccia, P. (1983) Intensive melphalan chemotherapy and cryopreserved autologous bone marrow transplantation for the treatment of refractory cancer. Journal of Clinical Oncology 1(6):359-367. Abstract.

LeCluyse, E.L., Alexandre, E., Hamilton, G.A., Viollon-Abadie, C., Coon, D.J., Jolley, S., Richert, L. (2005) Isolation and culture of primary human hepatocytes. Methods in Molecular Biology 290:207-229. Abstract.

Lee, J.S., Chu, I.S., Heo, J., Calvisi, D.F., Sun, Z., Roskams, T., Durnez, A., Demetris, A.J., Thorgeirsson, S.S. (2004) Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. Hepatology 40(3):667-676.

Lee, W.M. (1995) Drug-induced hepatotoxicity. The New England Journal of Medicine 333(17):1118-1127.

Lee, W.M. (2003) Drug-induced hepatotoxicity. The New England Journal of Medicine 349(5):474-485.

Leist, M., Single, B., Castoldi, A.F., Kuhnle, S., Nicotera, P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. Journal of Experimental Medicine 185(8):1481-1486.

Lekas, P., Tin, K.L., Lee, C., Prokipcak, D. (2000) The human cytochrome P450 1A1 mRNA is rapidly degraded in HepG2 cells. Archives of Biochemistry and Biophysics 384(2):311-318.

Lemasters, J.J. (1999) Mechanisms of hepatic toxicity. V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. The American Journal of Physiology 276:G1-6.

Leroy, I., Laurent, G., Quillet-Mary, A. (2006) Mithramycin A activates death pathways in leukaemic cell lines. Apoptosis 11:113-119.

Levin, S. (1998) Apoptosis, necrosis or oncosis: what is your diagnosis? A report from the cell death nomenclature committee of the society of toxicologic pathologists. Toxicological Sciences 41:155-156.

Li, A.P., Roque, M.A., Beck, D.J., Kaminski, D.L. (1992) Isolation and culturing of hepatocytes from human livers. Journal of Tissue Culture Methods 14:139-146.

Li, A.P., Lu, C., Brent, J.A., Pham, C., Fackett, A., Ruegg, C.E., Silber, P.M. (1999) Cryopreserved human hepatocytes: characterisation of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. Chemico-Biological interactions 121:17-35.

Li, A.P., Gorycki, P.D., Hengstler, J.G., Kedderis, G.L., Koebe, H.G., Rahmani, R., de Sousas, G., Silva, J.M., Skett, P. (1999) Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: consensus of an international expert panel. Chemico-Biological Interactions 121:117-123.

Li, A.P. (2007) Human hepatocytes: isolation, cryopreservation and applications in drug development. Chemico-biological interactions 168(1):16-29.

Limdi, J.K., Hyde, G.M. (2003) Evaluation of abnormal liver function tests. Postgraduate Medical Journal 79(932):307-312.

Link, K.H., Kornmann, M., Formentini, A., Leder, G., Sunelaitis, E.,Schatz, M., Pressmar, J., Beger, H.J. (1999) Regional chemotherapy of non-resectable liver metastases from colorectal cancer – literature and institutional review. Lagenbeck's Arhives of Surgery 384(4):344-353.

Lloyd, S., Hayden, M.J., Sakai, Y., Fackett, A., Silber, P.M., Hewitt, N.J., Li, A.P. (2002) Differential in vitro hepatotoxicity of troglitazone and rosglitazone among cryopreserved human hepatocytes from 37 donors. Chemico-Biological Interactions 142:57-71.

Longley, D., Harkin, D.P., Johnston, P. (2003) 5-fluorouracil: mechanisms of action and clinical strategies. Nature Reviews Cancer 3:330-338.

Lu, Y., Cederbaum, A.I. (2006) Cisplatin-induced hepatotoxicity is enhanced by elevated expression of cytochrome P450 2E1. Toxicological Sciences 89(2):515-523.

Macpherson, J.S., Jodrell, D.I., Guichard, S.M. (2006) Validation of real-time reverse-transcription-polymerase chain reaction for quantification of capecitabine-metabolizing enzymes. Analytical Biochemistry 350:71-80.

Majno, G., Joris, I. (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. American Journal of Pathology 146(1):3-15.

Malerba, I., Casati, S., Diodovich, C., Parent-Massin, D., Gribaldo, L. (2004) Inhibition of CFU-E/BFU-E and CFU-GM colony growth by cyclophosphamide, 5-fluorouracil and taxol: development of a high-throughput in vitro method. Toxicology in vitro 18(3):293-300.

Manov, I., Hirsh, M., Iancu, T.C. (2002) Acetaminophen hepatotoxicity and mechanisms of its protection by N-acetylcysteine: a study of Hep3B cells. Experimental and Toxicologic Pathology 53(6): 489-500.

Martignoni, M., de kanter, R., Grossi, P., Saturno, G., Barbaria, E., Monshouwer, M. (2006) An in vivo and in vitro comparison of CYP gene induction in mice using liver slices and quantitative RT-PCR. Toxicology in Vitro 20:125-131.

Martin, D.S., Bertino, J.R., Koutcher, J.A. (2000) ATP depletion + pyrimidine depletion can markedly enhance cancer therapy: fresh insight for a new approach. Cancer Research 60:6776-6783.

Marzolini, C., Paus, E., Buclin, T., Kim, R.B. (2004) Polymorphisms in human MDR1 (p-glycoprotein): recent advances and clinical relevance. Clinical Pharmacology and Therapeutics 75(1): 13-33.

Masubuchi, N., May, R.D., Atsumi, R. (2004) A predictive model of human myelotoxicity using five camptothecin derivatives and the *in vitro* colony-forming unit granulocyte/macrophage assay. Clinical Cancer Research 10:6722-6731.

Matsumura, M., Ijichi, M., Shiratori, Y., Yogo, G., Hikiba, Y., Inoued, K., Kohara, M., Omata, M. (2001) Simple quantitative assay of alpha-fetoprotein mRNA in liver tissue using the real-time detection polymerase chain reaction assay – its application in clinical use. Hepatology Research 20:84-96.

McIntosh, S., Davidson, D.L., O'Brien, R.T., Pearson, H.A. (1977) Methotrexate hepatotoxicity in children with leukaemia. The Journal of Pediatrics 90(6):1019-1021. Abstract.

Mersch-Sundermann, V., Knasmuller, S., Wu, X-J., Darroudi, F., Kassie, F. (2004) Use of human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. Toxicology 198:329-340.

Meurette, O., Lefeuvre-Orfila, L., Rebillars, A., Lagadic-Gossmann, D., Dimanche-Boitrel, M-T. (2005) Role of intracellular glutathione in cell sensitivity to the apoptosis induced by tumour necrosis factor α-related apoptosis-inducing ligand/anticancer drug combinations. Clinical Cancer Research 11(8) 3075-3083.

Miller, D.M., Polansky, D.A., Thomas, S.D., Ray, R., Campbell, V.W., Sanchez, J., Koller, C.A. (1987) Mithramycin selectively inhibits transcription of G-C containing DNA. The American Journal of Medical Sciences 294(5):388-394. Abstract.

Minchenko, A., Leshchinsky, I., Opentanova, I., Sang, N., Srinivas, V., Armstead, V., Caro, J. (2002) Hypoxia-inducible factor-1 mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. The Journal of Biological Chemistry 277(8):6183-6187.

Miret, S., De Groene, E.M., Klaffke, W. (2006) Comparison of in vitro assays of cellular toxicity in the human hepatic cell line HepG2. Journal of Biomolecular Screening 11(2):184-193.

Mitry, R.R., Hughes, R.D., Dhawan, A. (2002) Progress in human hepatocytes: isolation, culture and cryopreservation. Cell and Developmental Biology 13:463-467.

Moronvalle-Halley, V., Sacre-Salem, B., Sallez, V., Labbe, G., Gautier, J.C. (2005) Evaluation of cultured, precision-cut rat liver slices as a model to study drug-induced liver apoptosis. Toxicology 207(2):203-214. Abstract.

Morris, L.E., Guthrie, T.H. Jr. (1988) Busulfan-induced hepatitis. American Journal of Gastroenterology 83(6):682-683. Abstract.

Moss, D.W. (1997) Physiochemical and pathophysiological factors in the release of membrane-bound alkaline phosphatase from cells. Clinica chimica acta 257(1): 133-140.

Nagata, T. (2006) Electron microscopic radioautographic study on protein synthesis in hepatocyte mitochondria of aging mice. The Scientific World Journal 6:1583-1598.

Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T., Sato, J. (1982) Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Research 42:3858-3863.

Nakagawa, K., Miller, F.N., Sims, D.E., Lentsch, A.B., Miyazaki, M., Edwards, M.J. (1996) Mechanisms of interleukin-2-induced hepatic toxicity. Cancer Research 56:507-510.

Nakata, K., Tanaka, Y., Nakano, T., Adachi, T., Tanaka, H., Kaminuma, T., Ishikawa, T. (2006) Nuclear-receptor mediated transcriptional regulation in phase I, II, and III xenobiotic metabolizing systems. Drug Metabolism and Pharmacokinetics 21(6): 437-457.

Navarro, V.J., Senior, J.R. (2006) Drug-related hepatotoxicity. The New England Journal of Medicine 354:731-739.

Nelson, D.R. (2006) Cytochrome P450 nomenclature, 2004. Methods in Molecular Biology 320:1-10.

Newell, D.R., Burtles, S.S. Fox, B.W., Jodrell, D.I., Connors, T.A. (1999) Evaluation of rodent-only toxicology for early clinical trials with novel cancer therapeutics. British Journal of Cancer 81(5):760-768.

Nicotera, P., Leist, M., Ferrando-May, E. (1998) Intracellular ATP, a switch in the decision between apoptosis and necrosis. Toxicology Letters 102-103: 139-142.

Nishimura, M., Yokoi, T., Tateno, C., Kataoka, M., Takahashi, E., Horie, T., Yoshizato, K., Naito, S. (2005) Induction of human CYP1A2 and CYP3A4 in primary culture of hepatocytes from chimeric mice with humanized liver. Drug Metabolism and Pharmacokinetics 20(2):121-126.

O'Brien, P.J., Irwin, W., Diaz, D., Howard-Cofield, E., Krejsa, C.M., Slaughter, M.R., Gao, B., Kaludercic, N., Angeline, A., Bernardi, P., Brain, P., Hougham, C. (2006) High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Archives of Toxicology 80(9):580-604.

O'Brien, T., Babcock, G., Cornelius, J., Dingeldein, M., Talaska, G., Warshawsky, D., Mitchell, K. (2000) A comparison of apoptosis and necrosis induced by hepatotoxins in HepG2 cells. Toxicology and Applied Pharmacology 164(3):280-290.

Oettle, H., Arnold, D., Esser, M., Huhn, D., Riess, H. (2000) Paclitaxel as a weekly second-line therapy in patients with advanced pancreatic carcinoma. Anticancer Drugs 11(8): 635-638. Abstract.

Olinga, P., Meijer, D.K.F., Slooff, M.J.H., Groothuis, G.M.M. (1997) Liver slices in in vitro pharmacotoxicology with special reference to the use of human liver tissue. Toxicology in Vitro 12:77-100.

Olinga, P., Merema, M., Hof, I.H., De Jong, K.P., Slooff, M.J.H., Meijer, D.K.F., Groothuis, G.M.M. (1998) Effect of human liver source on the functionality of isolated hepatocytes and liver slices. Drug metabolism and Disposition 26(1):5-11.

Onaitis, M., Morse, M., Hurwitz, H., Cotton, P., Tyler, D., Clavien, P., Clary, B. (2003) Adjuvant hepatic arterial chemotherapy following metastasectomy in patients with isolated liver metastases. Annals of Surgery 237(6):782-788.

Osborne, N.J., Tonkin, A.L., Miners, J.O. (1991) Interethnic differences in drug glucuronidation: a comparison of paracetamol metabolism in Caucasians and Chinese. British Journal of Clinical Pharmacology 32(6):765-767. Abstract.

Ostrowska, A., Bode, D.C., Pruss, J., Bilir, B., Smith, G.D., Zeisloft, S. (2000) Investigation of functional and morphological integrity of freshly isolated and cryopreserved human hepatocytes. Cell Tissue Bank 1(1):55-68.

Paciucci, P.A., Sklarin, N.T. (1986) Mitoxantrone and hepatotoxicity. Annals of Internal Medicine 105:805-806.

Paine, A.J., Andreakos, E. (2004) Activation of signalling pathways during hepatocyte isolation: relevance to toxicology in vitro. Toxicology in Vitro 18:187-193.

Papazisis, K.T., Geromichalos, G.D., Dimitriadis, K.A., Kortsaris, A.H. (1997) Optimization of the sulforhodamine B colorimetric assay. Journal of Immunological Methods 208:151-158.

Park, B.K., Kitteringham, N.R., Maggs, J.L., Pirmohamed, M., Williams, D.P. (2005) The role of metabolic activation in drug-induced hepatotoxicity. Annual Review of pharmacology and toxicology 45:177-202.

Pascussi, J-M., Drocourt, L., Gerbal-Chaloin, S., Fabre, J-M., Maurel, P., Vilarem, M-J. (2001) Dual effects of dexamethasone on CYP3A4 gene expression in human hepatocytes. European Journal of Biochemistry 268:6346-6357.

Peppercorn, P.D., Reznek, R.H., Wilson, P., Slevin, M.L., Gupta, R.K. (1998) Demonstration of hepatic steatosis by computerised tomography in patients receiving 5-fluorouracil-based therapy for advanced colorectal cancer. British Journal of Cancer 77(11): 2008-2011.

Perlmutter, D.H. (2002) Liver injury in α-1 antitrypsin deficiency: an aggregated protein induces mitochondrial injury. The Journal of Clinical Investigation 110:1157-1583.

Persad, R., Liu, C., Wu, T-T., Houlihan, P.S., Hamilton, S.R., Diehl, A.M., Rashid, A. (2004) Overexpression of caspase-3 in hepatocellular carcinomas. Modern pathology 17:861-867.

Pessina, A., Albella, B., Bayo, M., Bueren, J., Brantom, P., Casati, S., Croera, C., Gagliardi, G., Foti, P., Parchment, R., Parent-Massin, D., Schoeters, G., Sibiril, Y., Van Den Heuvel, R., Gribaldo, L. (2003) Application of the CFU-GM Assay to predict acute drug-induced neutropenia: an international blind trial to validate a prediction model for the maximum tolerated dose (MTD) of myelosuppressive xenobiotics. Toxicological Sciences 75:355-367.

Petak, I., Houghton, J.A. (2001) Shared pathways: death receptors and cytotoxic drugs in cancer therapy. Pathology and Oncology Research 7(2): 95-106.

Pfeifer, A.M.A., Cole, K.E., Smoot, D.T., Weston, A., Groopman, J.D., Shields, P.G., Vignaud, J-M., Juillerat, M., Lipsky, M.M., Trump, B.F., Lechner, J.F., Harris, C.C. (1993) Simian virus 40 large tumor antigen-immortalized normal human liver epithelial cells express hepatocyte characteristics and metabolize chemical carcinogens. Proceedings of the National Academy of Sciences USA 93:5123-5127.

Pingpank, J.F., Libutti, S.K., Chang, R., Wood, B.J., Neeman, Z., Kam, A.W., Figg, W.D., Zhai, S., Beresneva, T., Seidel, G.D., Alexander, H.R. (2005) Phase I study of hepatic arterial melphalan infusion and hepatic venous hemofiltration using percutaneously placed catheters in patients with unresectable hepatic malignancies. Journal of Clinical Oncology 23(15) 6435-3474.

Pinkoski, M.J., Brunner, T., Green, D.R., Lin, T. (2000) Fas and Fas ligand in gut and liver. The American Journal of Physiology 278:G354-366.

Pinto, H.C., Baptista, A., Camilo, M.E., de Costa, E.B., Valente, A., de Moura, M.C. (1995) Tamoxifen-associated steatohepatitis – report of 3 cases. Journal of Hepatology 23(1):95-97. Abstract.

Pizzuto, J., Aviles, A., Ramos, E., Cervera, J., Aguirre, J. (1983) Cytosine arabinoside induced liver damage: histopathologic demonstration. Medical and Pediatric Oncology 11(4):287-290.

Poelstra, K., Bakker, W.W., Klok, P.A., Hardonk, M.J., Meijer, D.K. (1997) A physiological function for alkaline phosphatase: endotoxin detoxification. Laboratory Investigation 76(3): 319-327.

Poso, A., Honkakoski, P. (2006) Ligand recognition by drug-activated nuclear receptors PXR and CAR: structural, site-directed mutagenesis and molecular modeling studies. Mini Reviews in Medicinal Chemistry 6(8):937-947.

Pritchard, J., Raine, J., Wallendszus, K. (1989) Hepatotoxicity of actinomycin-D. Lancet 1(8630):168.

Propper, D.J., Saunders, M.., Salisbury, A.J., Long, L., O'Byrne, K.J., Braybrooke, J.P., Dowsett, M., Taylor, M., Talbot, D.C., Ganesan, T.S., Harris, A.L. (1999) Phase I study of the novel cyclic AMP (cAMP) analogue 8-chloro-cAMP in patients with cancer: toxicity, hormonal, and immunological effects. Clinical Cancer Research 5:1682-1689.

Pumford, N.R., Halmes, N.C. (1997) Protein targets of xenobiotic reactive intermediates. Annual Review of Pharmacology and Toxicology 37:91-117.

Quesada, J.R., Taipaz, M., Rios, A., Kurzrock, R., Gutterman, J.U. (1986) Clinical toxicity of interferons in cancer patients: a review. Journal of Clinical Oncology 4(2):234-243. Abstract.

Raber, M.N., Newman, R.A., Newman, B.M., Gaver, R.C., Schacter, L.P. (1992) Phase I trial and clinical pharmacology of elsamitrucin. Cancer Research 52(6):1406-1410.

Radonic, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., Nitsche, A. (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochemical and Biophysical Research Communications 313:856-862.

Raida, M., Schwabe, W., Hausler, P., Van, Kuilenburg, A.B., Van Gennip, A.H., Behnke, D., Hoffken, K. (2001) Prevalance of a common point mutation in the dihydropyrmidine dehydrogenase (DPD) gene with the 5'-splice donor site of intron 14 in patients with severe 5-fluorouracil (5-FU)-related toxicity compared with controls. Clinical Cancer Research 7(9):2832-2839.

Rang, H.P., Dale, M.M., Ritter, J. (2001) Pharmacology 5th edition. Churchill Livingstone. Pages 40-62.

Raucy, J.L. (2003) Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. Drug Metabolism and Disposition 31(5) 533-539.

Riss, T.L., Moravec, R.A. (2004) Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay and Drug Development Technologies 2(1):51- 62.

Roberts , R.A., Ganey, P.A., Ju, C., Kamendulis, L.M., Rusyn, I., Klaunig, J.E. (2007) Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. Toxicological Sciences 96(1):2-15.

Robinson, K., Lambiase, L., Li, J., Monteiro, C., Schiff, M. (2003) Fatal cholestatic liver failure associated with gemcitabine therapy. Digestive Diseases and Sciences 48(9):1804-1808.

Rodriguez-Antona, C., Ingelman-Sundberg, M. (2006) Cytochrome P450 pharmacogenetics and cancer. Oncogene 25(11):1679-1691.
Rosenthal, K.L., Tompkins, W.A., Frank, G.L., McCulloch, P., Rawls, W.E. (1977) Variants of a human colon adenocarcinoma cell line which differ in morphology and carcinoembryonic antigen production. Cancer Research 37(11):4024-4030.

Rosser, B.G., Gores, G.J. (1995) Liver cell necrosis: cellular mechanisms and clinical implications. Gastroenterology 108:252-275. Abstract.

Rowinsky, E.K., Noe, D.A., Ettinger, D.S., Christian, M.C., Lubejko, B.G., Fishman, E.K., Sartorius, S.E., Boyd, M.R., Donehower, R.C. (1993) Phase I and pharmacological study of the pulmonary cytotoxin 4-ipomeanol on a single dose schedule in lung cancer patients: hepatotoxicity is dose-limiting in humans. Cancer Research 53(8):1794-1801.

Roymans, D., Van Looveren, C., Leone, A., Parker, B.J., Mcmillian, M., Johnson, M.D., Koganti, A., Gilssen, R., Silber, P Mannens, G., Meuldermans, W. (2004) Determination of cytochrome P450 1A2 and cytochrome P450 3A4 in cryopreserved human hepatocytes. Biochemical Pharmacology 67(3):427-437.

Rubbia-Brandt, L., Audard, V., Sartoretti, P., Roth, A.D., Brezault, C., Le Charpentier, M., Dousset, B., Morel, P., Soubrane, O., Chaussade, S., Mentha, G., Terris, B. (2004) Severe hepatic sinusoidal obstruction associated with oxaliplatin-based chemotherapy in patients with metastatic colorectal cancer. Annals of Oncology 15:460-466.

Ryan, D.P., Supko, J.G., Eder, J.P., Seiden, M.V., Demetri, G., Lynch, T.J., Fischman, A.J., Davis, J., Jimeno, J., Clark, J.W. (2001) Phase I and pharmacokinetic

study of ecteinascidin 743 administered as a 72-hour continuous intravenous infusion in patients with solid malignancies. Clinical Cancer Research 7:231-242.

Sachs, B., Haider, S., Balaraman, R., Shahab, N., Perry, M.C. (2002) Hepatotoxicity of chemotherapy. Expert opinion on drug safety 1(4):339-353.

Samali, A., Nordgren, H., Zhivotovsky, B., Peterson, E., Orrenius, S. (1999) A comparative study of apoptosis and necrosis in HepG2 cells: oxidant-induced caspase inactivation leads to necrosis. Biochemical and Biophysical Research Communications 255:6-11.

Satti, M.B., Weinbren, K., Gordon-Smith, E.C. (1982) 6-thioguanine as a cause of toxic veno-occlusive disease of the liver. Journal of Clinical Pathology 35(10):1086-1091.

Schmittgen, T.D., Zakrajsek, B.A. (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. Journal of Biochemical and Biophysical Methods 46:69-81.

Seglen, P.O. (1976) Preparation of isolated rat liver cells. Methods in Cell Biology 13:29-83.

Semple, T.U., Quinn, L.A., Woods, L.K., Moore, G.E. (1978) Tumour and lymphoid cell lines from a patient with carcinoma of the colon for a cytotoxicity model. Cancer Research 38(5):1345-1355.

Sewell, J.M., Mayer, I., Langdon, S.P., Smyth, J.F., Jodrell, D.I., Guichard, S.M. (2005) The mechanism of action of Kahalalide F: variable cell permeability in human hepatoma cell lines. European Journal of Cancer 41(11):1637-1644.

Simons, K., Fuller, S.D. (1985) Cell surface polarity in epithelia. Annual Review of Cell Biology 1:243-288.

Skehan, P., Storang, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the National Cancer Institute 82(13):1107-1112.

Sonna, L.A., Fujita, J., Gaffin, S.L., Lilly, C.M. (2002) Molecular biology of thermal regulation. Invited review: Effects of heat and cold stress on mammalian gene expression. Journal of Applied Physiology 92:1725-1742.

Sonna, L.A., Cullivan, M.L., Sheldon, H.K., Pratt, R.E., Lilly, C.M. (2003) Effect of hypoxia on gene expression by human hepatocytes (HepG2). Physiological Genomics 12:195-207.

Spicker, J.S., Pedersen, H.T., Nielsen, H.B., Brunak, S. (2007) Analysis of cell death inducing compounds. Archives of Toxicology 81(11):803-811.

Stacey, G., MacDonald, C. (2001) Immortalisation of primary cells. Cell biology and toxicology 17(4-5):231-246.

Stoff-Khalili, M.A., Rivera, A.A., Le, L.P., Stoff, A., Everts, M., Contreras, J.L., Chen, D., Teng, L., Rots, M.G., Haisma, H.J., Rocconi, R.P., Bauerschmitz, G.J., Rein, D.T., Yamamoto, M., Siegal, G.P., Dall, P., Mathis, J.M., Curiel, D.T. (2006) Employment of liver tissue slice analysis to assay hepatotoxicity linked to replicative and nonreplicative adenoviral agents. Cancer Gene Therapy 13(6):606-618.

Stragand, J.J., Barlogie, B., White, A., Drewinko, B. (1981) Biological properties of the human colonic adenocarcinoma cell line SW 620 grown as a xenograft in the athymic mouse. Cancer Research 41:3364-3369.

Supko, J.G., Eder, J.P., Ryan, D.P., Seiden, M.V., Lynch, T.J., Amrein, P.C., Kufe, D.W., Clark, J.W. (2003) Phase I clinical trial and pharmacokinetic study of spicamycin analog KRN5500 administered as a 1-hour intravenous infusion for five consecutive days to patients with refractory solid tumors. Clinical Cancer Research 9:5178-5186.

Temple, R. (2006) Hy's Law: predicting serious hepatotoxicity. Pharmacoepidemiology and Drug Safety 15:241-243.

Terry, C., Dhawan, A., Mitry, R.R., Lehec, S.C., Hughes, R.D. (2005) Preincubation of rat and human hepatocytes with cryoprotectants prior to cryopreservation can improve viability and function upon thawing. Liver Transplantation 12:165-177.

Terry, C., Mitry, R.R., Lehec, S.C., Muiesan, P., Rela, M., Heaton, N.D., Hughes, R.D., Dhawan, A. (2005) The effects of cryopreservation on human hepatocytes obtained from different sources of liver tissue. Cell Transplantation 14(8):585-594.

Thomson, A.B.R., Shaffer, E.A. (2000) First Principles of Gastroenterology: The basis of disease and an approach to management. 5th Edition: pages 491-500.

Thummel, K.E., Wilkinson, G.R. (1998) In vitro and in vivo drug interactions involving human CYP3A. Annual Reviews in Pharmacology and Toxicology 38:389-430.

Tietz, P, Larusso, N.F. (2002) Cholangiocyte biology. Current Opinion in Gastroenterology 18(3): 360-365.

Timsit, Y., E., Negishi, M. (2007) CAR and PXR: the xenobiotic-sensing receptors. Steroids 72(3): 231-246.

Tirmenstein, M.A., Hu, C.X., Gales, T.L., Maleeff, B.E., Narayanan, P.K., Kurali, E., Hart, T.K., Thomas, H.C., Schwartz, L.W. (2002) Effects of troglitazone on HepG2 viability and mitochondrial function. Toxiclogical Sciences 69:131-138.

Tisman, G., MacDonald, D., Shindell, N., Reece, E., Patel, R., Honda, N., Nishimora, E.K., Garris, J., Shannahan, W., Christi, N., McCarthy, J., Moaddeli, S.N., Sargent, D., Plant, A. (2004) Oxaliplatin toxicity masquerading as recurrent colon cancer. Journal of Clinical Oncology 22(15):3202-3204.

Tokyol, C., Karaorman, G., Bastug, M. (2005) Effects of acute and adaptive hypoxia on heat shock protein expression in hepatic tissue. High Altitude Medicine and Biology 6(3):247-255.

Tran, A., Housset, C., Boboc, B., Tourani, J.M., Carnot, F., Berthelot, P. (1991) Etoposide (VP 16-213) induced hepatitis. Report of three cases following standard-dose treatments. Journal of Hepatology 12(1):36-39. Abstract.

Trauner, M., Meier, P.J., Boyer, J.L. (1998) Molecular pathogenesis of cholestasis. The New England Journal of Medicine 339(17): 1217-1227.

Tsujimoto, Y. (1997) Apoptosis and necrosis: intracellular ATP levels as a determinant for cell death modes. Cell Death and Differentiation 4:429-434.

Uetrecht, J. (2007) Idiosyncratic Drug Reactions: Current Understanding. Annual Review of Pharmacology and Toxicology 47:513-539.

Ulrich, R.G., Bacon, J.A., Cramer, C.T., Peng, G.W., Petrella, D.K., Stryd, R.P., Sun, E.L. (1995) Cultured hepatocytes as investigational models for hepatic toxicity: practical applications in drug discovery and development. Toxicology Letters 82/83:107-115.

Underwood, J.C., Shahani, R.T., Blackburn, E.K. (1970) Cholestatic jaundice following treatment of chronic granulocytic leukaemia with busulphan. Journal of Clinical Pathology 23(9):827. Abstract.

Underwood, J.C., Shahani, R.T., Blackburn, E.K. (1971) Jaundice after treatment of leukaemia with busulphan. British Medical Journal 1(5748):556-557. Abstract.

Usui, T., Saitoh, Y., Komada, F. (2003) Induction of CYP3As in HepG2 cells by several drugs. – Association between induction of CYP3A4 and expression of glucocorticoid receptor -. Biological and Pharmaceutical Bulletin 26(4):510-517.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3(7):research0034.1-0034.11.

van Schaik, R.H. (2005) Cancer treatment and pharmacogenetics of cytochrome P450 enzymes. Investigational New Drugs 23(6): 513-522.

Vastag, B. (2006) HSP-90 inhibitors promise to complement cancer therapies. Nature Biotechnology 24(11): 1307.

Vermill, M., Judson, I. (1994) Jaundice with ondansetron. Lancet 344:190-191.

Wang, K., Shindoh, H., Inoue, T., Horii, I. (2002) Advantages of in vitro cytotoxicity testing by using primary rat hepatocytes in comparison with established cell lines. The Journal of Toxicological Sciences 27(3):229-237.

Wanwimolruk, S., Bhawan, S., Coville, P.F., Chalcroft, S.C. (1998) Genetic polymorphism of debrisoquine (CYP2D6) and proguanil (CYP2C19) in South Pacific Polynesian populations. Abstract.

Weil, M., Jacobson, M.D., Coles, H.S.R. et al. (1996) Constitutive expression of the machinery for programmed cell death. Journal of Cell Biology 133: 1053-1059.

Wessels, H.P., Geffen, I., Spiess, M. (1989) A hepatocyte-specific basolateral membrane protein is targeted to the same domain when expressed in Madin-Darby canine kidney cells. The Journal of Biological Chemistry 264(1):17-20.

Whitlock, J.P.Jr. (1999) Induction of cytochrome P4501A1. Annual Review of Pharmacology and Toxicology 39:103-125.

Wilkening S., Stahl, F., Bader, A. (2003) Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. Drug Metabolism and Disposition 31(8):1035-1042.

Witte, R.S., Cnaan, A., Mansour, E.G, Barylak, E., Harris, J.E., Schutt, A.J. (2001) Comparison of 5-fluorouracil alone, 5-fluorouracil with levamisole, and 5-fluorouracil with hepatic irradiation in the treatment of patients with residual, non-measurable, intra-abdominal meastasis after undergoing resection for colorectal carcinoma. Cancer 91(5):1020-1028.

Wysowski, D.K., Freiman, J.P., Tourtelot, J.B., Horton, M.L. (1993) Fatal and nonfatal hepatotoxicity associated with flutamide. Annals of Internal Medicine 118(11):860-864.

Xu, J.J., Diaz, D., O'Brien, P.J. (2004) Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. Chemico-Biological Interactions 150:115-128.

Yamamoto, Y., Kume, M., Yamaoka, Y. (1998) Implications of heat shock proteins during liver surgery and liver perfusion. Recent Results in Cancer Research 147:157-172.

Yamashita, T., Hashimoto, S., Kaneko, S., Nagai, S., Toyoda, N., Suzuki, T., Kobayashi, K., Matsushima, K. (2000) Comprehensive gene expression profile of a normal human liver. Biochemical and Biophysical Research Communications 269(1):110-116.

Yang, M.S., Yu, L.C., Gupta, R.C. (2004) Analysis of changes in energy and redox states in HepG2 hepatoma and C6 glioma cells upon exposure to cadmium. Toxicology 201:105-113.

Yang, T-S., Lin, Y-C., Chen, J-S., Wang, H-M., Wang, C-H. (2000) Phase II study of gemcitabine in patients with advanced hepatocellular carcinoma. Cancer 89(4):750-756.

Zalcberg, J., Kerr, D., Seymour, L., Palmer, M. (1998) Haematological and non-haematological toxicity after 5-fluorouracil and leucovorin in patients with advanced colorectal cancer is significantly associated with gender, increasing age and cycle number. European Journal of Cancer 34(12):1871-1875.

Zhang, H., Forman, H.J., Choi, J. (2005) Gamma-glutamyl transpeptidase in glutathione biosynthesis. Methods in Enzymology 401:468-483.

Zheng, J-F., Wang, H-D. (2005) 5-Fluorouracil concentration in blood, liver and tumor tissues and apoptosis of tumor cells after pre-operative oral 5`-deoxy-5-fluorouridine in patients with hepatocellular carcinoma. World Journal of Gastroenterology 11(25):3944-3947.

Zimmerman, H.J. (1999) Hepatotoxicity: The adverse effects of drugs and other chemicals on the liver. 2nd Edition. (ed. Zimmerman, H).

Zojer, N., Keck, A.V., Pecherstorfer, M (1999) Comparative tolerability of drug therapies for hypercalcaemia of malignancy. Drug Safety 21(5):389-406.

Zorzi, D., Laurent, A., Pawlik, T.M., Lauwers, G.Y., Vauthey, J.N., Abdalla, E.K. (2007) Chemotherapy-associated hepatotoxicity and surgery for colorectal liver metastases. The British Journal of Surgery 94(3):274-286.

http:// www.ecacc.org.uk [Accessed 17 April 2007]

http://ctep.cancer.gov/forms/CTCv20_4-30-992.pdf [Accessed 13 March 2007]

http://www.cancerbackup.org.uk [Accessed 13 March 2007]

http://www.britishlivertrust.org.uk [Accessed 14 March 2007]

]http://dtp.nci.nih.gov/docs/cancer/cancer_data.html [Accessed 20 June 2007]

http://www.celsis.com/filelib/CryoHepMTTTox.pdf [Accessed 23 November 2007]

Appendix A: Published Papers

Guichard, S., Else, R., **Reid, E.**, Zeitlin, B., Aird, R., Muir, M., Dodds, M., Fiebig, H., Sadler, P., Jodrell, D. (2006) Anti-tumour activity in non-small cell lung cancer models and toxicity profiles for novel ruthenium(II) based organo-metallic compounds. Biochemical Pharmacology 71(4):408-415

BIOCHEMICAL PHARMACOLOGY 71 (2006) 408–415

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Anti-tumour activity in non-small cell lung cancer models and toxicity profiles for novel ruthenium(II) based organo-metallic compounds

S.M. Guichard [a], R. Else [b], E. Reid [a], B. Zeitlin [a], R. Aird [a], M. Muir [a], M. Dodds [a], H. Fiebig [d], P.J. Sadler [c], D.I. Jodrell [a,*]

[a] Pharmacology and Drug Development Group, Cancer Research UK Centre, University of Edinburgh, Western General Hospital, Crewe Road South, Edinburgh EH4 2XR, UK
[b] Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Veterinary Centre, Easter Bush, Roslin, Midlothian EH25 9RG, UK
[c] School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, UK
[d] Institute for Experimental Oncology, Oncotest GmbH, Am Flughafen 12-4, D-79108 Freiburg, Germany

ARTICLE INFO

Article history:
Received 29 July 2005
Accepted 18 October 2005

Keywords:
Ruthenium(II) organo-metallic complex
Lung cancer
Hepatotoxicity

ABSTRACT

Novel ruthenium(II) organo-metallic compounds are active in ovarian cancer models [Aird RE, Cummings J, Ritchie AA, Muir M, Morris RE, Chen H, et al. In vitro and in vivo activity and cross resistance profiles of novel ruthenium(II) organometallic arene complexes in human ovarian cancer. Br J Cancer 2002;86(10):1652–7]. $[(\eta^6\text{-}C_6H_5C_6H_5)Ru(en)Cl]^+$ (as a PF_6 salt, where en = ethylenediamine (RM175)) has been evaluated in a 13-cell line panel. Particular sensitivity (~10-fold lower than mean IC_{50}) was noted in breast cancer and non-small cell lung cancer cell lines. In addition, IC_{50} in the A549 was 2 μM and RM175 (25 mg kg^{-1}, days 1 and 5, i.p.) caused a significant ($p = 0.004$) growth delay in a xenograft model. HC11 [(η^6-tetrahydroanthracene)Ru(en)Cl]PF_6 was more potent in the A549 cell line (IC_{50} 0.5 μM). HC11 (25 mg kg^{-1}, days 1, 8 and 15, i.p.) was also active in vivo. Following RM175 25 mg kg^{-1}, days 1 and 5, and 15 mg kg^{-1}, days 1–5, HC11 25 and 40 mg kg^{-1}, day 1, elevated alanine transaminase levels were detected, suggesting hepatotoxicity. No changes were observed in kidney or haematological parameters. In liver sections, multi-focal hepatic necrosis was seen, becoming confluent at high doses of HC11. In vitro studies confirmed that HC11 was more toxic than RM175 to fresh human hepatocytes and equitoxic to mithramycin. Liver toxicity may be related to the arene ligand and modification may reduce the potential for hepatic toxicity, while maintaining the anti-tumour activity seen.

1. Introduction

The metal ruthenium (Ru) possesses several favourable chemical properties that indicate it may be a strong candidate to form a basis for rational anticancer drug design [2,3]. Ru^{II} complexes demonstrate similar ligand exchange kinetics to those of platinum (Pt^{II} and Pt^{IV}) while displaying only low toxicity. Ruthenium(III) has the ability to mimic iron in binding to plasma proteins including transferrin and albumin [3]. Transport and sequestration of Ru into tumour cells may be mediated via protein transport and receptor mediated uptake [4,5]. Due to differing ligand geometry between their

* Corresponding author. Tel.: +44 131 777 3512; fax: +44 131 777 3520.
E-mail address: duncan.jodrell@cancer.org.uk (D.I. Jodrell).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2005.10.053

complexes, some Ru^{III} compounds (octahedral) bind to DNA forming predominately inter-strand crosslinks as opposed to the intra-strand crosslinks favoured by square-planar Pt^{II} in cisplatin [6,7]. In addition, non-nuclear targets, such as the mitochondrion and the cell surface, have also been implicated in the antineoplastic activity of Ru complexes, particularly in the case of the clinically investigated Ru^{III} antimetastatic drug *trans*-[$RuCl_4$(DMSO)(Im)](ImH) (NAMI-A) [8]. NAMI-A has completed clinical phase I evaluation, and cutaneous toxicity was dose-limiting. Nausea and vomiting was also noted. Mild reversible nephrotoxicity was noted and a pre- and post-hydration regimen was utilised. Neurotoxicity and hepatic toxicities were not identified. Stable disease was identified in a patient with non-small cell lung cancer.

Previous investigators have focused on Ru^{III} complexes as potential anti-tumour agents [9–11]. These are thought to be activated by reduction to Ru^{II} in the body (Clarke et al. [2]). In contrast, we have developed a series of novel organo-metallic Ru^{II} arene complexes [12] including [(η^6-$C_6H_5C_6H_5$)Ru(en)Cl]$^+$ (RM175) and [(η^6-tetrahydroanthracene)Ru(en)Cl]$^+$ (HC11). The presence of the arene ligand stabilises the Ru^{II} oxidation state [13]. These Ru^{II} complexes have been evaluated for activity in both in vitro and in vivo models of human ovarian cancer, and cross-resistance profiles established in cisplatin and multi-drug resistant (MDR) variants [1]. Issues relevant to the cytotoxicity of these compounds are their selectivity for guanine on DNA, leading to the formation of an N7–Ru bond together with the formation of a strong hydrogen bond between the C6 carbonyl group of guanine and an NH proton of the ethylenediamine ligand.

In this paper we report results of studies to assess the anti-tumour activity in a range of tumour types using an automated screen approach and subsequent studies focusing on non-small cell lung cancer models. We also report preliminary toxicity data, to illustrate the likely therapeutic index of these compounds.

2. Materials and methods

RM175 and HC11 (Fig. 1) were synthesised and characterised using the methods reported previously [12,14]. A549 and H520 cell lines were obtained from Cancer Research UK Cell Services. Fresh human hepatocytes were obtained from the UK Human Tissue Bank after ethical approval from NHS Lothian.

2.1. *In vitro cytotoxicity using propidium iodide fluorescence assay*

Human tumour cells (see Table 1) were plated in 96-well flat-bottomed microtitre plates (50 μL cell suspension, 1×10^5 or 5×10^4 cells/mL) and additional 50 μL of culture medium was added. After a 24 h recovery, 50 μL of culture medium containing 50 μg/mL gentamycin was added into the six control wells or medium containing the test drug was added to the wells. Each drug concentration was plated in triplicate. Following 3–6 days of incubation, depending on cell doubling time, a modified propidium iodide (PI) assay [15] was performed. Culture medium was replaced by fresh medium and 50 μL of an aqueous propidium iodide solution (25 μg/mL) was added to each well. PI does not cross intact cell membranes and enters only the nucleus of dead cells by intercalation into DNA and RNA. The fluorescence signal correlates with the number of dead cells. Fluorescence (FU_1) was measured using a Millipore Cytofluor 2350 microplate reader (excitation 530 nm, emission 620 nm). Microplates were then kept at −18 °C for 24 h, yielding in a total cell kill. After thawing of the plates and a second fluorescence measurement (FU_2) the amount of viable cells was calculated by subtraction of FU_2 from FU_1.

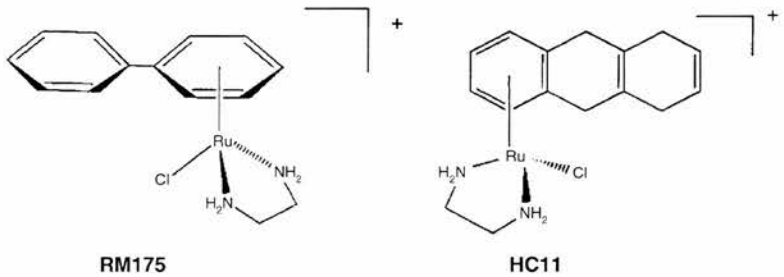
Growth inhibition was expressed as treated/control × 100 (%T/C); inhibiting concentrations (IC) were determined by plotting compound concentration versus cell viability. Mean IC_{50}, IC_{70} and IC_{90} values were calculated for each individual cell line. From all IC_{70} values the mean IC_{70} was calculated according to the formula:

$$\text{Mean } IC_{50,70} = 10^{\frac{(\sum_1^n \log(IC_{50,70})_x)}{n}}$$

where x is the value of specific tumour cell line and n is the total number of tumour cell lines studied. If an IC_{50} or IC_{70} could not be determined within the examined dose range, the lowest or highest concentration studied was used for the calculation.

2.2. *In vitro cytotoxicity in A549 and H520 using sulforhodamine B assay*

The cytotoxic activity of the test compounds was assessed using a cell death assay based on detection of cells by sulforhodamine B (SRB), a stain specific for proteins, used for this purpose by the United States National Cancer Institute [16]. Briefly, A549 and H520 were plated out at densities,



RM175 HC11

Fig. 1 – Chemical structures of HC11 and RM175.

Table 1 – In vitro growth inhibition in lung cancer cell lines

	A549		H520	
	IC50 (μM)	Range	IC50 (μM)	Range
RM175	3.0	2.9–3.3	3.5	3–3.8
HC11	0.50	0.49–0.51	0.53	0.53–0.54
CDDP	2.6	2.9–4.2	9.5	9.3–9.8

determined by growth curve analysis (data not shown), that gave the best range of log phase growth after 2–3 days culture in 96-well plates. After this time the media were removed from each well by aspiration and fresh media containing drug at known concentrations were added to each well. Cells were incubated a further 24 h and the media removed by aspiration. Wells were washed in warmed PBS (200 μL) and then layered with fresh culture media. Cells were then incubated 72 h, at 37 °C/5% CO_2. To each well was then added trichloroacetic acid (TCA), 10% final concentration and the plates were incubated 1 h at 4 °C in order to fix the cells. Wells were washed gently under running water and allowed to air dry. SRB, 50 μL, was then added to each well and the plates incubated 30 min at room temperature. The excess SRB was then washed off with four washes of 1% acetic acid and the plates allowed to completely air dry. To each well was then added Tris–HCl, pH 10.5, 150 μL/well and the plates were incubated 1 h at room temperature and then very gently mixed by shaking. Absorbance was read at 540 nm on a 96-well microplate reader.

2.3. *In vitro cytotoxicity in fresh human hepatocytes using the MTT assay*

Fresh human hepatocytes (20,000 viable cells) were plated in collagen-I pre-coated 96-well plates (BD Biosciences, Oxford, UK), in William's E medium (WEM) (Cambrex, Wokingham, UK) supplemented with 10% FCS, L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), 10 nM insulin and 30 nM dexamethasone and allowed to adhere for 12 h at 37 °C/5% CO_2 in a humidified atmosphere. Stock solutions of HC11 and RM175 were prepared with 5% (v/v) dimethylsulfoxide (DMSO) so that the final concentration of DMSO the hepatocytes were exposed to was never greater then 0.5%. A stock solution of mithramycin (Sigma) was prepared using sterile water. Cells were treated with 50 μL of RM175 (1–50 μM), HC11 (0.07–13.5 μM) or mithramycin (0.1–10 μM) for 24 h. At the end of drug exposure, the media were aspirated and the cells washed with 50 μL PBS before adding 200 μL/well of fresh complete WEM. Cytotoxicity studies were performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay 24 h after the end of drug treatment. Cells were incubated in 200 μL fresh 37 °C WEM containing 0.4 mg/mL MTT (Sigma, Gillingham, UK) for 3 h in the dark at 37 °C. Medium was then removed, and formazan crystals were solubilised in 200 μL DMSO. The optical density was measured at 570 nm using a Biohit BP800 microplate reader. A comparison between the MTT and SRB assays was also undertaken (data not shown) to ensure relevant and valid comparison of IC_{50} values obtained using both assays.

2.4. *In vivo toxicity of RM175 and HC11*

These studies were performed in C57/Bl 6 mice 6-weeks old. Treatment schedules for RM175 administration were: RM175 schedule 1—25 mg kg^{-1} administered i.p. days 1 and 5 (50 mg kg^{-1} total); RM175 schedule 2—15 mg kg^{-1} administered i.p. days 1–4 (60 mg kg^{-1} total). For HC11, the following schedules were used: HC11 schedule 1—25 mg kg^{-1} administered i.p. on day 1; HC11 schedule 2—40 mg kg^{-1} administered i.p. on day 1. The following parameters were measured in plasma: haematology (RBC, WBC and platelets) and biochemistry (ALT, ALP, total bilirubin, creatinine and urea) and the liver, spleen, lungs, kidneys and peritoneum were collected for subsequent evaluation. Three animals were to be culled at each of the following time-points: RM175 schedule 1—days 3, 5, 8 and 10; RM175 schedule 2—days 2–5; HC11 schedules 1 and 2—days 2–5.

2.5. *Pathology studies*

Liver and spleen from experimental and control animals were removed immediately after sacrifice and fixed by immersion in 10% buffered formalin. Liver lobes were embedded in paraffin wax and sectioned at 5 μm for histopathological assessment. All sections were stained with haematoxylin and eosin (H&E) using standard techniques. Sections were scanned and assessed for significant changes on two occasions. Selected liver sections were examined for fatty change using oil-red-O stain.

2.6. *In vivo efficacy of RM175 in LXFL xenografts*

RM175 was tested for in vivo activity in the large cell lung cancer model LXFL 529 growing subcutanously in serial passage in NMRI-nude mice. LXFL 529 was derived from a patient with untreated large cell lung cancer. Master stocks were frozen down at passage 7 in between liquid nitrogen and these experiments were performed in passage 24. The characteristics of the models have been described previously [17].

Nu/nu athymic mice of NMRI background from an in-house breeding facility were used for all experiments. Tumours were implanted s.c. in both flanks of 6-week-old nude mice. Treatment commenced when tumours reached diameters of 5–6 mm. Animals were randomly assigned into treatment groups. Food and water were provided ad libitum. Tumour growth was followed by serial two-dimensional caliper measurements and body weight documented concomitantly twice a week. Tumour volumes were calculated according to the formula (length × $width^2$)/2 and mean as well as median relative tumour volume (vol. day x/day 0 × 100) were used for analyses. Data were presented as mean relative tumour volumes ± S.E., optimal T/C values of the test divided by the control relative tumour volumes were calculated.

2.7. *In vivo efficacy of RM175 and HC11 in A549 xenografts*

Xenografts were established from the A549 cell lines by subcutaneous implantation of 10^7 cells in serum free media in the flank of animals. All animal experiments were carried out

according to UKCCCR guidelines [18]. Female nu/nu mice were implanted on both flanks with 2–3 mm^3 fragments of viable tumour and randomised when tumour volumes reached 30–100 mm^3. Animals were randomised into control and drug treated groups (5 animals/group). Ru^{II} complexes were administered as 10% DMSO solutions in sterile saline at a volume of 0.1 mL/10 g of body weight i.p. HC11 was administered at a dose of 25 mg kg^{-1} on days 1, 8 and 15. RM175 was administered at a dose of 25 mg kg^{-1} on days 1 and 5. Tumour xenografts were measured three times a week in perpendicular diameters and tumour volume calculated as (length × $width^2$)/2.

3. Results

3.1. *In vitro testing of RM175 in the 14 cell line panel*

RM175, $[(\eta^6\text{-}C_6H_5C_6H_5)Ru(en)Cl]^+$ (as a PF_6 salt), was shown to have a mean IC_{70} of 3.9 μg/mL (~8 μM) and the IC_{50} value was 3 μM, confirming the level of activity we have reported previously (Aird et al. [1]) and demonstrating it has a broad spectrum of activity (Fig. 2). Particular sensitivity (~10-fold lower than mean IC_{50}) was noted in a breast cancer cell line (401NL) and a non-small cell lung cancer (NSCLC) cell line (LXFL 529L). Comparative analyses were performed to ascertain whether the pattern of sensitivity correlated with other known agents. The strongest correlation was with mitoxantrone (0.61), but this was not thought to be significant, considering the number of cell lines tested (13).

The cytotoxicity of both compounds was also determined in two other non-small cell lung cancer cell lines, H520 and A549 (Table 1). HC11 $[(\eta^6\text{-tetrahydroanthracene})Ru(en)Cl]PF_6$ was highly cytotoxic in both cell lines with IC_{50}'s of 0.53 and 0.5 μM, respectively. RM175 was less active than HC11 with IC_{50}'s of 3 and 3.5 μM in A549 and H520, respectively, but similar to cisplatin (IC_{50} = 2.6 and 9.5 μM for A549 and H520, respectively).

3.2. *In vivo toxicity of RM175 and HC11*

In order to evaluate the activity of both compounds, the determination of a non-toxic dose was necessary. For RM175, the previously determined active dose of 25 mg kg^{-1} on days 1 and 5 and a more dose intense schedule of 15 mg kg^{-1} day^{-1} × 5 were evaluated. Two dose levels, 25 and 40 mg kg^{-1}, 1, were tested for HC11, administered on day 1 only.

Weight loss of 10% was observed after administration of 15 mg kg^{-1} day^{-1} of RM175 on day 5 but this did not have a significant impact on the animals. In contrast, HC11 administered at a dose of 40 mg kg^{-1} induced significant weight loss with all animals losing 10% body weight by day 3 (range 5–16%). Animals were unwell and were culled earlier than the proposed days 5 and 8 time-points.

The evaluation of blood biochemistry parameters showed a rise in alanine transaminase (ALT), noted following the administration of HC11, in particular (Fig. 3). Following a single dose of 25 mg kg^{-1}, i.p., the elevation of ALT was maximal at 48 h (mean 418 U L^{-1} in the treated group, mean 97 U L^{-1} in the control groups) and had resolved by day 8. Following

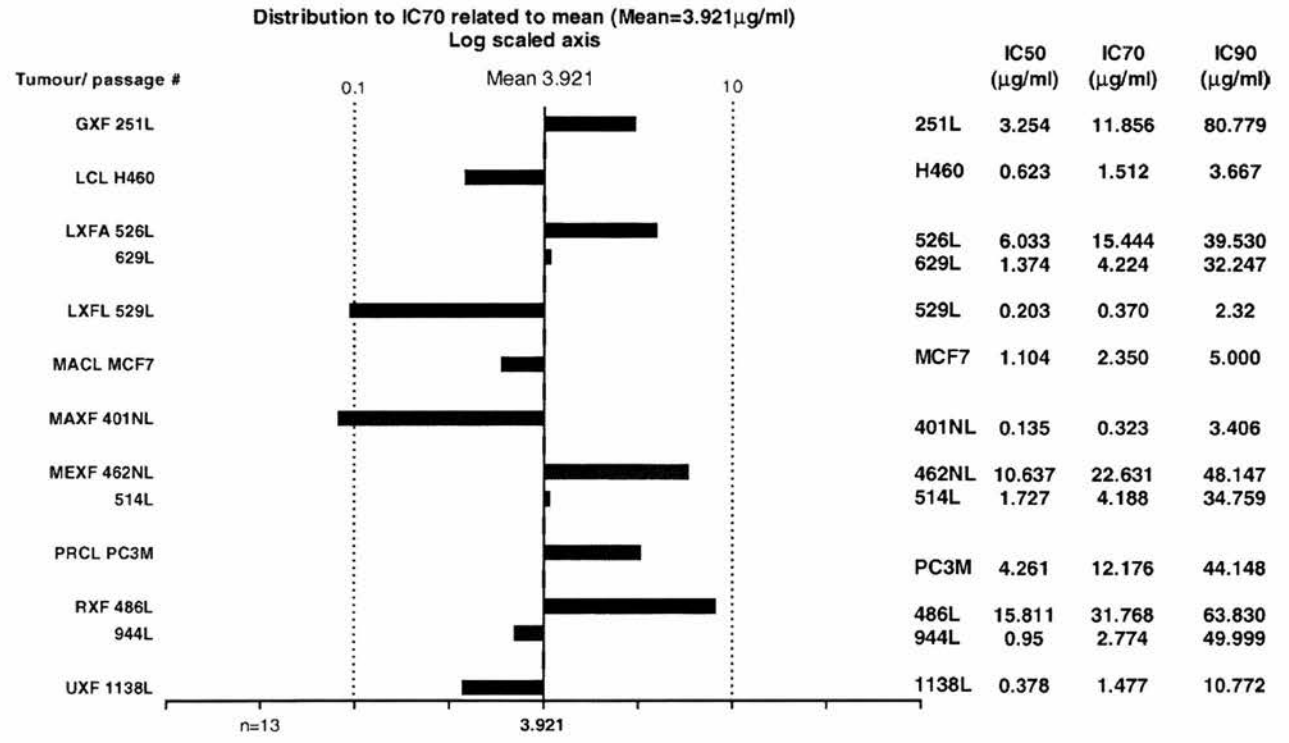


Fig. 2 – In vitro data from the 13 cell lines in the Freiburg screen. IC_{70} data (μg/mL) are expressed relative to the mean IC_{70} (3.921 μg/mL).

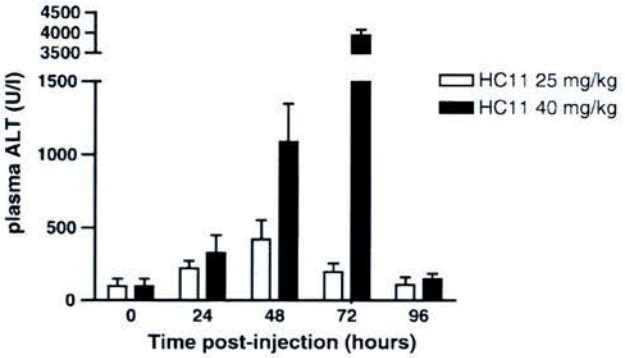


Fig. 3 – Changes in alanine transaminase (ALT) plasma levels in animals following administration of HC11 at the dose of 25 mg kg^{-1} (white bars) or 40 mg kg^{-1} (black bars) as a single injection i.p. on day 1. Results are mean ± S.D. of three animals at each time-point.

40 mg kg^{-1}, there was a marked elevation of ALT by 72 h (mean 3907 U L^{-1}) compared to untreated animals (mean 97 U L^{-1}). These changes were associated with histopathological changes (Fig. 4): following treatment with HC11 (25 mg kg^{-1}), on day 2, no major differences were identified between treated and control animals, although there was a low level of fatty-like vacuolar change around large blood vessels (portal tracts) in the treated group. However, on days 3 and 5 the occurrence of multiple small and larger foci of necrosis of hepatocellular parenchyma accompanied by infiltrations of polymorphs (neutrophils) and macrophages was noted. In the animals sacrificed on day 8, a similar lesion was identified in 1/3 animals and showed features suggestive of scarring. At all time-points, in animals receiving 40 mg kg^{-1}, there was a variable but often severe focal or more diffuse/lobar necrotising reaction with polymorph and macrophage presence. These infiltrations were either parenchymal or, more often, perivascular. Individual apoptotic hepatocytes in the adjacent hepatocellular parenchyma were also seen in many of these livers. Where lobar lesions were present the adjacent lobes were usually unaffected, however. The lesions were more marked in the animals culled on days 4 and 5.

Following administration of RM175, elevation of ALT was seen in the group receiving 15 mg kg^{-1} on days 1–5 after 96 h (mean 355 U L^{-1}) compared to control animals (62 U L^{-1}). No elevation of ALT was seen in the group receiving 25 mg kg^{-1} on days 1 and 5. The histopathology showed only minor changes such as small polymorph and macrophage inflammatory foci adjacent to the hilar region in 2/3 animals on day 4. The significance of this is unclear, as low-level inflammatory cells were present in the extrahepatic omental hilar tissue in many of the sections in the series, but were not seen in control animals. A solitary peripherally located small necrotic focus with a few associated polymorphs was seen in 1/3 animals on day 5. The cause of this was not apparent, but it is similar, but less severe than the changes associated with HC11.

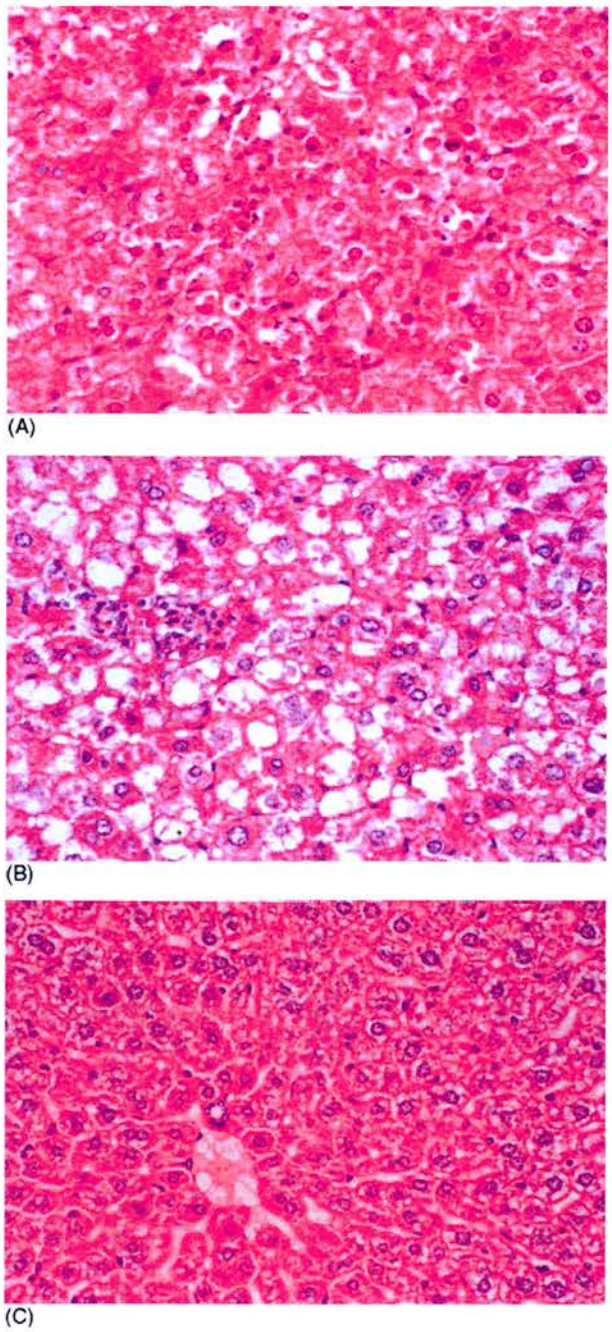


Fig. 4 – Microscopic evidence of liver toxicity after HC11 administration. Animals were treated with HC11 i.p. on day 1. Livers were collected on day 3 and pathology examination was carried out after H&E staining. (A) HC11 40 mg kg^{-1} showing extensive necrosis; (B) HC11 25 mg kg^{-1} showing vacuolar change, consistent with fatty degeneration; (C) control.

No changes were noted in urea or creatinine over the course of the experiment. A single animal in the high dose (40 mg kg^{-1}) HC11 study was noted to be thrombocytopenic, but this occurred on day 5 when the animal was profoundly unwell.

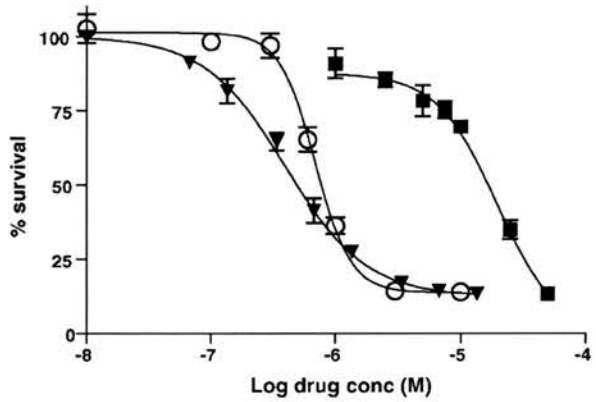


Fig. 5 – In vitro cytotoxicity of RM175 (■), HC11 (▼) and mithramycin (○) in fresh human hepatocytes. Cells were treated over a range of concentrations with RM175, HC11 or mithramycin for 24 h. Twenty-four hours after the end of drug treatment, the MTT assay was performed as described. Results are expressed as percentage of survival as compared to untreated cells and are the mean ± S.E.M of triplicate samples.

3.3. *In vitro cytotoxicity of HC11 and RM 175 in fresh human hepatocytes*

To ascertain whether the changes noted in the in vivo experiments in rodents might be relevant in patients, we evaluated the cytotoxicity of RM175 and HC11 in vitro in fresh human hepatocytes, using the known hepatotoxic drug, mithramycin, as a positive control. Both ruthenium compounds induced a rapid cytotoxic effect (Fig. 5), detectable at the end of drug exposure (24 h). IC_{50} values for the two ruthenium compounds were markedly different, with HC11 being 40-fold more toxic (RM175 IC_{50} 24 μM, HC11 IC_{50} 0.6 μM). In comparison, the IC_{50} for mithramycin was 0.7 μM.

3.4. *In vivo anti-tumour activity of RM175 and HC11*

The anti-tumour activity of RM175 and HC11 was evaluated using 25 mg kg^{-1}, days 1 and 5 of RM175 and 25 mg kg^{-1} of HC11 on days 1, 8 and 15. RM175 was also evaluated at two dose levels in the LXFL 529L xenografts, in Freiburg. Both RM175 and HC11 were evaluated in the A549 model. Significant growth delay was noted in the A549 model (Fig. 6A) on day 46 for both RM175 ($V_t/V_0 = 5.3 \pm 1.9$, $p = 0.004$) and HC11 ($V_t/V_0 = 4.7 \pm 0.7$, $p = 0.001$) compared to controls (9.8 ± 1.1). However, anti-tumour activity was not seen in the LXFL 529L model (Fig. 6B), although this study was performed in the NMRI mouse strain and only 4% body weight loss was noted (data not shown), so possibly below the maximally tolerated dose.

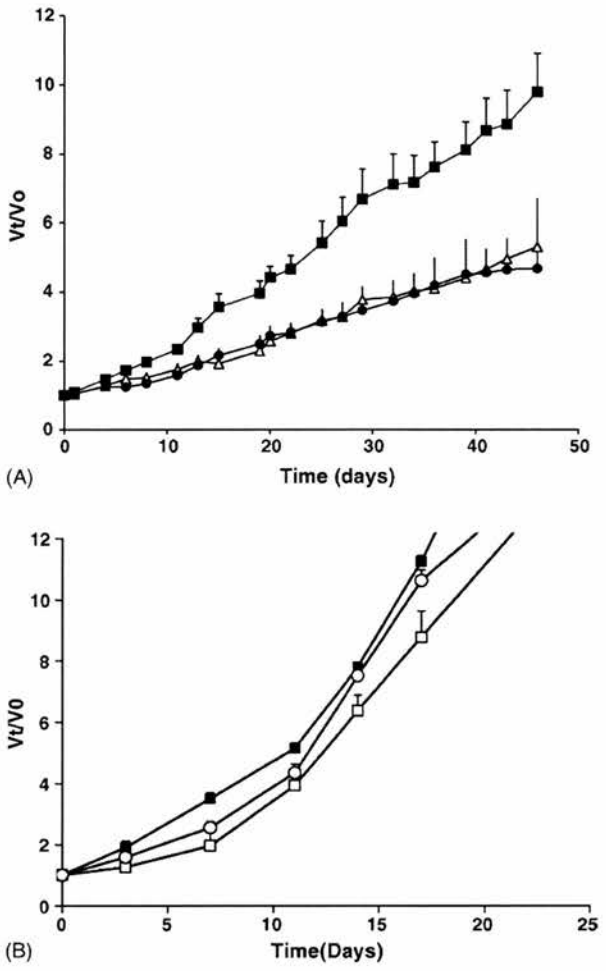


Fig. 6 – In vivo efficacy of RM175 and HC11 on A549 (A) and LXFL lung cancer xenografts (B). (A) Animals bearing A549 xenografts were treated with RM175 25 mg kg^{-1}, i.p., on days 1 and 5 (●), HC11 25 mg kg^{-1} on days 1, 8 and 15 (△) or vehicle (■). (B) Animals bearing LXFL xenografts received vehicle (■) or RM175 i.p. on days 1, 5 and 13 at the dose of 15 mg kg^{-1} (○) or 25 mg kg^{-1} (□). Results are expressed as the average growth rate (V_t/V_0) of each group ± S.E.

4. Discussion

This paper presents the anti-tumour activity and safety profiles of two Ru^{II} arene complexes, RM175 and HC11, in vitro and in vivo. In vitro assessment of RM175, $[(\eta^6\text{-}C_6H_5C_6H_5)Ru(en)Cl]^+$ (as a PF_6 salt) using the Freiburg screen, demonstrated a mean IC_{50} of 3 μM, confirming the level of activity we have reported previously and demonstrating RM175 has a broad spectrum of activity. Particular sensitivity (~10-fold lower than mean IC_{50}) was noted in breast cancer (401NL) and non-small cell lung cancer (529L) cell lines. Using the data from the Freiburg screen, it was not possible to identify compounds with a similar spectrum of activity, or to identify a particular mechanism of action, i.e. RM175 was "compare-negative" in this screen.

The particular activity (RM175 $IC_{50} < 0.5$ μM) noted in the large cell lung cancer model, in vitro, in the Freiburg screen, led us to undertake further in vitro studies in two additional non-small cell lung cancer cell lines, A549 and H520. The

IC_{50} values were similar to those obtained in the Freiburg screen for RM175 which confirmed the activity seen in lung cell lines. Moreover, HC11 was more active than RM175 and cisplatin in these two cell lines. HC11 has been shown previously to be 10-fold more active than RM175 in the ovarian cancer cell line, A2780 [1]. These results are in contrast with the lack of cytotoxicity of NAMI-A observed in vitro against breast and colon cancer cell lines [19] and suggest a different cellular target for these two classes of compounds. These Ru^{II} arene compounds have been shown to interact strongly with guanine bases in DNA [14].

To provide a preliminary assessment of the potential therapeutic index for these agents, we evaluated myelosuppression, liver and renal toxicity, as possible sequelae of the use of a heavy metal containing anti-proliferative complex. Previous pharmacokinetic studies using NAMI-A reported high concentrations of ruthenium in the kidneys after a single i.v. administration [20]. When NAMI-A was administered daily i.p., the highest concentrations of ruthenium were observed in the liver and kidneys [21]. In this study, no clear evidence of either bone marrow or kidney toxicity was identified. However, liver toxicity was observed with both RM175 and HC11. It was mild and reversible in animals receiving RM175 and HC11 at low doses (25 mg kg^{-1}) but became severe in animals treated with HC11 at a dose of 40 mg kg^{-1}, suggesting a low therapeutic index for this particular compound.

Fresh hepatocyte models have been used previously to investigate liver toxicity and are considered to be one of the best in vitro models for this purpose [22]. Mithramycin is a known hepatotoxic anti-cancer drug [23] and has been included as a positive control in this study with fresh hepatocytes. All three compounds (HC11, RM175 and mithramycin) caused hepatocyte toxicity after drug treatment. However, HC11 was 40-fold more cytotoxic (IC_{50} 0.6 μM) than RM175 (IC_{50} 24 μM) and equitoxic to the known hepatotoxin, mithramycin (IC_{50} 0.7 μM). Moreover, the IC_{50} for RM175 was approximately 10-fold higher in fresh hepatocytes, when compared to the IC_{50} observed for A549 and H520 (see Table 1), suggesting a log difference between drug concentrations active in tumour cells and concentrations toxic for normal cells. However, HC11 displayed similar IC_{50} values in fresh hepatocytes and in NSCLC cell lines, suggesting a low (~1) therapeutic index. It is unlikely that the cytotoxic effect observed in vitro is due to the opening of the arene ring in the tissue culture medium since previous studies have shown the stability of these ruthenium complexes in presence of amino acids, peptides, proteins and nucleotides [24–26].

The anti-tumour activity of both RM175 and HC11 was evaluated in animals bearing A549 xenograft and RM175 was also evaluated in Freiburg in the LXFL lung model. Both RM175 and HC11 were active in the A549 model, but RM175 did not show activity in the LXFL model. Pharmacokinetic studies of NAMI-A have reported an excellent distribution in the lungs [21]. This was associated with an antimetastatic effect of this compound against Lewis lung carcinoma in mice. A preferential uptake of ruthenium by lung cells may also be suggested considering the anti-tumour activity of NAMI-A against Lewis lung carcinoma grown as xenografts [21].

Hepatotoxicity is not a recognised side effect following the administration of platinum containing drugs, at standard dosages, and was not a feature of the NAMI-A phase I trial. In a study of the administration of high-dose carboplatin, reversible biochemical hepatotoxicity was identified, but doses six times higher than the standard dosage regimen were being used [27]. Titanocene dichloride is also an organo-metallic compound containing cyclic aromatic hydrocarbons (cyclopentadienyl). It was associated with reversible liver and GI tract damage, observed for 4–8 days after administration at 40–60 mg kg^{-1} in pre-clinical studies [28]. In subsequent phase I trials, renal toxicity was the major dose-limiting event, although hepatotoxicity was dose-limiting in one patient [29,30]. It is known that cyclic aromatic hydrocarbons can be hydroxylated and conjugated in the liver leading to the generation of potentially toxic species. Therefore, studies to evaluate the role of cytochrome P450s in induction of liver toxicity are being performed and these will be incorporated into screening protocols, if appropriate. The present study suggests that fresh human hepatocytes can be used to evaluate the potential hepatotoxicity of this class of agents and that indeed HC11 may be potentially hepatotoxic. However, further chemical synthesis is ongoing to "design out" this toxicity and improve the therapeutic index of this series of compounds.

Acknowledgements

We thank the Edinburgh Technology Fund and Oncosense Ltd. for their support for this work and Drs. Robert Morris, Haimai Chen and Abraha Habtemariam for the synthesis of compounds.

REFERENCES

[1] Aird RE, Cummings J, Ritchie AA, Muir M, Morris RE, Chen H, et al. In vitro and in vivo activity and cross resistance profiles of novel ruthenium(II) organometallic arene complexes in human ovarian cancer. Br J Cancer 2002;86(10):1652–7.
[2] Clarke MJ, Zhu F, Frasca DR. Non-platinum chemotherapeutic metallopharmaceuticals. Chem Rev 1999;99(9):2511–34.
[3] Allardyce CS, Dyson PJ, Coffey J, Johnson N. Determination of drug binding sites to proteins by electrospray ionisation mass spectrometry: the interaction of cisplatin with transferring. Rapid Commun Mass Spectrom 2002;16(10):933–5.
[4] Bergamo A, Messori L, Piccioli F, Cocchietto M, Sava G. Biological role of adduct formation of the ruthenium(III) complex NAMI-A with serum albumin and serum transferring. Invest New Drugs 2003;21(4):401–11.
[5] Pongratz M, Schluga P, Jakupec M, Arion V, Hartinger C, Allmaier G, et al. Transferrin binding and transferrin-mediated cellular uptake of the ruthenium coordination compound KP1019, studied by means of AAS, ESI-MS and CD spectroscopy. J Anal At Spectrom 2004;19(1):46–51.
[6] Fruhauf S, Zeller WJ. New platinum, titanium, and ruthenium complexes with different patterns of DNA damage in rat ovarian tumor cells. Cancer Res 1991;51(11):2943–8.
[7] Gallori E, Vettori C, Alessio E, Vilchez FG, Vilaplana R, Orioli P, et al. DNA as a possible target for antitumor

ruthenium(III) complexes. Arch Biochem Biophys 2000;376(1):156–62.
[8] Bergamo A, Zorzet S, Gava B, Sorc A, Alessio E, Iengo E, et al. Effects of NAMI-A and some related ruthenium complexes on cell viability after short exposure of tumor cells. Anticancer Drugs 2000;11(8):665–72.
[9] Keppler BK, Balzer W, Seifried V. Synthesis and antitumor activity of triazolium-bis(triazole)-tetrachlororuthenate (III) and bistriazolium-triazolepentachlororuthenate (III). Two representatives of a new class of inorganic antitumor agents. Arzneimittelforschung 1987;37(7):770–1.
[10] Berger MR, Garzon FT, Keppler BK, Schmahl D. Efficacy of new ruthenium complexes against chemically induced autochthonous colorectal carcinoma in rats. Anticancer Res 1989;9(3):761–5.
[11] Seelig MH, Berger MR, Keppler BK. Antineoplastic activity of three ruthenium derivatives against chemically induced colorectal carcinoma in rats. J Cancer Res Clin Oncol 1992;118(3):195–200.
[12] Morris RE, Aird RE, Murdoch Pdel S, Chen H, Cummings J, Hughes ND, et al. Inhibition of cancer cell growth by ruthenium(II) arene complexes. J Med Chem 2001;44(22):3616–21.
[13] Melchart M, Sadler PJ. Ruthenium arene anticancer complexes. In: Jaouen G, editor. Bioorganometallics. Weinheim, Germany: Wiley/VCH, 2005.
[14] Chen H, Parkinson JA, Parsons S, Coxall RA, Gould RO, Sadler PJ. Organometallic ruthenium(II) diamine anticancer complexes: arene-nucleobase stacking and stereospecific hydrogen-bonding in guanine adducts. J Am Chem Soc 2002;124(12):3064–82.
[15] Dengler WA, Schulte J, Berger DP, Mertelsmann R, Fiebig HH. Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays. Anticancer Drugs 1995;6(4):522–32.
[16] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82(13):1107–12.
[17] Fiebig HH, Berger DP, Dengler WA, Wallbrecher E, Winterhalter BR. Combined in vitro/in vivo test procedure with human tumor xenografts for new drug development. Karger Verlag; 1992.
[18] Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double JA, et al. United Kingdom Co-Ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (2nd edition). Br J Cancer 1998;77:1–10.
[19] Zorzet S, Bergamo A, Cocchietto M, Sorc A, Gava B, Alessio E, et al. Lack of In vitro cytotoxicity, associated to increased G(2)-M cell fraction and inhibition of matrigel invasion, may predict in vivo-selective antimetastasis activity of ruthenium complexes. J Pharmacol Exp Ther 2000;295(3):927–33.
[20] Cocchietto M, Salerno G, Alessio E, Mestroni G, Sava G. Fate of the antimetastatic ruthenium complex ImH [*trans*-$RuCl_4$(DMSO)Im] after acute i.v. treatment in mice. Anticancer Res 2000;20(1A):197–202.
[21] Sava G, Clerici K, Capozzi I, Cocchietto M, Gagliardi R, Alessio E, et al. Reduction of lung metastasis by ImH [*trans*-$RuCl_4$(DMSO)Im]: mechanism of the selective action investigated on mouse tumors. Anticancer Drugs 1999;10(1):129–38.
[22] Gomez-Lechon MJ, Donato MT, Castell JV, Jover R. Human hepatocytes as a tool for studying toxicity and drug metabolism. Curr Drug Metab 2003;4(4):292–312.
[23] King PD, Perry MC. Hepatotoxicity of chemotherapy. Oncologist 2001;6(2):162–76.
[24] Wang F, Chen H, Parkinson JA, Murdoch Pdel S, Sadler PJ. Reactions of a ruthenium(II) arene antitumor complex with cysteine and methionine. Inorg Chem 2002;41(17):4509–23.
[25] Wang F, Bella J, Parkinson JA, Sadler PJ. Competitive reactions of a ruthenium arene anticancer complex with histidine, cytochrome c and an oligonucleotide. J Biol Inorg Chem 2005;10(2):147–55 [Epub February 26, 2005].
[26] Chen H, Parkinson JA, Morris RE, Sadler PJ. Highly selective binding of organometallic ruthenium ethylenediamine complexes to nucleic acids: novel recognition mechanisms. J Am Chem Soc 2003;125(1):173–86.
[27] Shea TC, Flaherty M, Elias A, Eder JP, Antman K, Begg C, et al. A phase I clinical and pharmacokinetic study of carboplatin and autologous bone marrow support. J Clin Oncol 1989;7(5):651–61.
[28] Kopf-Maier P, Kopf H. Organometallic titanium, vanadium, niobium, molybdenum and rhenium complexes—early transition metal antitumour drugs. In: Metal compounds in cancer therapy. London: Chapman and Hall; 1994.
[29] Korfel A, Scheulen ME, Schmoll HJ, Grundel O, Harstrick A, Knoche M, et al. Phase I clinical and pharmacokinetic study of titanocene dichloride in adults with advanced solid tumors. Clin Cancer Res 1998;4(11):2701–8.
[30] Mross K, Robben-Bathe P, Edler L, Baumgart J, Berdel WE, Fiebig H, et al. Phase I clinical trial of a day-1, -3, -5 every 3 weeks phase I clinical trial of day-1, -3, -5 every 3 weeks schedule with titanocene dichloride (MKT 5) in patients with advanced cancer (Phase I Study Group of the AIO of the German Cancer Society). Onkologie 2000;23(6):576–9.

Guichard, S.M., Macpherson, J.S., Mayer, I., **Reid, E.**, Muir, M., Dodds, M., Alexander, S., Jodrell, D.I. (2007) Gene expression predicts differential capecitabine metabolism, impacting on both pharmacokinetics and antitumour activity. European Journal of Cancer doi:10.1016/j.ejca.2007.10.023

EUROPEAN JOURNAL OF CANCER 44 (2008) 310–317

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Gene expression predicts differential capecitabine metabolism, impacting on both pharmacokinetics and antitumour activity

Sylvie M. Guichard, Janet S. Macpherson, Iain Mayer, Eilidh Reid, Morwenna Muir, Michael Dodds, Susan Alexander, Duncan I. Jodrell*

Cancer Research UK Pharmacology and Drug Development Group, Edinburgh Cancer Research Centre, University of Edinburgh, Edinburgh, UK

ARTICLE INFO

Article history:
Received 27 August 2007
Received in revised
form 12 October 2007
Accepted 29 October 2007

Keywords:
Colorectal cancer
Capecitabine
Metabolism
Gene expression
Pharmacokinetics

ABSTRACT

Capecitabine is converted into 5′-deoxy-5-fluorocytidine (5′DFCR), 5′-deoxy-5-fluorouridine (5′DFUR) and 5-fluorouracil (5-FU) by CES1 and 2, CDD, and TP, in both liver and tumour. 5-FU is catabolised by DPD. Gene expression analysis of these enzymes was undertaken in fresh human hepatocytes, mouse liver, colorectal cancer cell lines and xenografts. Cell lines with low CDD expression (<1.5) had 5′DFCR/5′DFUR cytotoxicity ratios >2 and cell lines with TP/DPD < 0.6 had 5′DFUR IC_{50} > 50 μM (SRB assay). A pharmacokinetic/pharmacodynamic study in nude mice bearing HCT 116 xenografts and treated with capecitabine by oral gavage assessed pharmacokinetic, gene expression and antitumour activity. Low liver CDD correlated with high 5′DFCR plasma concentrations in mice. CDD expression was ~100-fold higher in fresh human hepatocytes than mouse liver, explaining the higher plasma 5′DFUR concentrations reported previously in humans. Tumour 5-FU concentration correlated with TP/DPD and with tumour response. These studies identify the potential utility of gene expression analysis and drug monitoring in tumour in patients.

1. Introduction

Capecitabine (Xeloda®; Hoffmann La Roche) is an oral fluoropyrimidine which mimics a continuous infusion of 5-fluorouracil (5-FU).[1–3] Capecitabine is metabolised in the liver and tumour by carboxylesterases (CES1 and CES2)[4] and cytidine deaminase (CDD) to 5′-deoxy-5-fluorocytidine (5′DFCR) and 5′-deoxy-5-fluorouridine (5′DFUR), respectively. 5′DFUR is subsequently activated by pyrimidine phosphorylases (thymidine phosphorylase (TP) and possibly uridine phosphorylase (UP)) by metabolism to 5-FU.[5,6] Dihydropyrimidine dehydrogenase (DPD), highly expressed in liver, metabolises 5-FU to dihydro 5-FU, inactivating it. In tumour cells, 5-FU interacts with its pharmacological target, thymidylate synthase (TS).

During capecitabine development, a physiologically-based pre-clinical model was built to predict the pharmacokinetics of capecitabine in patients.[7] This complex mathematical model was based on enzymatic activities of capecitabine metabolising enzymes determined *in vitro* in mouse tissues, limited pharmacokinetic data over 24 h after the administration of a single dose of capecitabine and physiological parameters (blood flow rate, volume of individual organs) based on the literature data. The model was shown to predict tumour exposure to 5-FU and integrated the interspecies differences between humans and mice. However, the population

* *Corresponding author:* Present address: Astra Zeneca, 11G76 Mereside, Alderley Park, Macclesfield SK10 4TF, UK. Tel.: +44 1625515289; fax: +44 1317773520.
E-mail address: sylvie.guichard@astrazeneca.com (S.M. Guichard).
0959-8049/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2007.10.023

pharmacokinetics studies carried out in patients including pharmacodynamic data (toxicity) did not show any clear relationship between systemic metabolite exposure in patients and toxicity, suggesting that drug and metabolite concentrations in plasma do not accurately reflect the tissues' exposure to the drug.[8,9] In another study, drug concentrations and enzyme activity in tumour, liver and plasma were determined in patients treated with capecitabine.[10] The study confirmed high concentrations of 5-FU in tumour tissue, but failed to establish a correlation between drug concentrations and enzyme activity.

Pre-clinical models using human cancer xenografts can address these issues. Due to the extensive metabolism of capecitabine, the levels of expression of the enzymes involved in its activation, both in liver and tumour, are likely to play a role in its antitumour effect. Indeed, overexpression of CDD in the T24 human bladder cancer cell line sensitised cells to 5′DFCR.[11] In xenograft tumours with high TP expression, higher tumour 5-FU concentrations were demonstrated after the administration of capecitabine than 5-FU suggesting tumour-specific activation of capecitabine by TP.[12–14] However, *in vitro*, similar 5′DFUR- and 5-FU-mediated cytotoxicity were observed in COLO320 cells (TP negative) and in WiDr cells (high TP), suggesting that UP might also metabolise 5′DFUR in the absence of TP.[15] Finally, to date, the expression of TS in tumours is the best predictive marker of response to 5-FU systemic therapy, but this has not been demonstrated for capecitabine.[16]

To develop a comprehensive view of the contribution of each enzyme to the metabolism of capecitabine, an *in vitro* study using 6 colon cancer cell lines was combined with a gene expression analysis of capecitabine-metabolising enzymes to identify key parameters for optimal cytotoxicity. A similar approach was also used *in vivo* in nude mice bearing the human colon cancer xenograft HCT 116. Capecitabine-metabolising enzymes gene expression was determined in xenograft tissue and mouse liver over a 3-week treatment period, using capecitabine at 2 dose levels. A similar gene expression analysis was carried out in human hepatocytes to identify interspecies differences in drug metabolism. Capecitabine and metabolite concentrations were also determined in liver, plasma and tumour xenografts.

2. Materials and methods

2.1. Cell lines and human hepatocytes

HCT 116, HCT8, HCT15, HT29, SW620 and COLO205 human colon cancer cells were obtained from the European Collection of Cell Cultures ECACC (Salisbury, UK). Fresh human hepatocytes from eight individual donors were obtained from the UK Human Tissue bank (Leicester, UK) after ethical approval from NHS Lothian (LREC/2003/8/42). Cells were received approximately 8 h after isolation. Only hepatocytes with viability greater than 80%, as determined by trypan blue exclusion at the time of collection, were used in the study. At the time of arrival, cells were centrifuged, resuspended in Tri-reagent® and stored at −80 °C until RNA extraction.

2.2. Gene expression analysis of capecitabine-metabolising enzymes by qRT-PCR

For cell lines, cells were collected in Tri-reagent® (SIGMA). For xenografts and liver samples, tissues were homogenised in Tri-reagent® (SIGMA) using a Mikro dismembrator (Sartorius, Epsom UK). Total RNA was prepared according to manufacturer's instructions followed by DNAse-treatment with Turbo-Free® DNAse (Ambion, Huntingdon UK). The quality of RNA was evaluated by electrophoretic analysis on a 2100 Agilent Bioanalyser. All qRT-PCR experiments were carried out with RNA integrity number >9. RNA concentrations were determined using a Nanodrop ND-1000. The transcripts of interest were amplified according to a previously published method.[17] Results were normalised by β-2-microglobulin (B2M) expression in both mouse liver and fresh hepatocytes, and by RNA polymerase II large subunit (POLR2) in HCT 116 xenografts (HCT 116 has a very similar expression for B2M and POLR2) and colon cancer cell lines. To compare gene expression in mouse and human tissues, a conversion factor was calculated between mouse and human standards used for quantification after verifying that all reaction efficiencies were close to 100% for all genes investigated. Three livers and six xenograft samples were analysed per time-point in each group unless the amount of RNA recovered from the tissue was insufficient for qRT-PCR. Gene expression is expressed as the ratios between the gene of interest (GOI) and the reference gene (REF).

2.3. In vitro cytotoxicity of capecitabine, 5′DFCR, 5′DFUR and 5-FU in human colon cancer cell lines

Drug concentrations that inhibited 50% of cell growth (IC_{50}) were determined using the sulforhodamine B technique.[18] Cells were plated on day 1 in 96-well plates at a density of 2500 cells/well for HCT 116, 3500 cells/well for HCT8 and HT29, 5000 cells/well for HCT15, 6000 cells/well for SW620 and 7000 cells/wells for COLO205 in a volume of 150 μl/well. All cell lines were treated on day 2 with increasing concentrations of capecitabine (0.1–10 mM), 5′DFCR (10 nM–100 μM), 5′DFUR (2.5–500 μM) or 5-FU (0.5–250 μM) for 24 h. After drug exposure, cells were washed once with cold PBS and placed in 200 μl of drug-free medium for 72 h after the end of drug exposure. The cells were then fixed with trichloroacetic acid and stained with sulforhodamine B. Optical densities were measured at 540 nm with a Biohit BP-800 (Bio-Hit, Helsinki, Finland). The results are based on three independent experiments performed in triplicate.

2.4. Pharmacokinetic/pharmacodynamic studies

Six-week-old C57/Bl6 Nu/Nu mice were obtained from Cancer Research UK (London, UK) and quarantined for 2 weeks before the start of experiments. Animal experiments were carried out under a project licence issued by the UK Home Office, and UKCCCR guidelines[19] were followed rigorously.

Bilateral HCT 116 xenografts were obtained by subcutaneous injection of 10^7 cells/flank. Animals bearing HCT 116 xenografts were treated with vehicle or capecitabine 0.52 or 2.1 mmol/kg (563 and 2250 mg/m^2, respectively) given once daily for 5 consecutive days/week by oral gavage for 3 weeks

(days 0–4, 7–11, 14–18). Animals were culled on day 0 at 15, 30 min, 1, 2, 4, 8 and 24 h, and prior to planned treatment on days 7 and 14 after the start of treatment. Three animals per time-point were analysed. At the time of collection, blood was collected in heparin, and plasma isolated and stored at −80 °C. The liver was removed immediately and stored in RNAlater® solution (SIGMA, Gillingham, UK). Tumours were macro-dissected to remove fibrotic tissue and blood vessels and snap-frozen in liquid nitrogen.

Tumour response was assessed during the course of treatment by caliper measurements of xenografts taken three times a week in perpendicular diameters from the start of treatment. Tumour volume was calculated as (length * width^2)/2. Results were expressed as mean ± SEM of tumour growth rate (ratio between volume on the day of measurements and tumour volume on the first day of drug treatment) of 16 and 10 xenografts for controls and treated animals, respectively.

2.5. *Determination of capecitabine and metabolite concentrations in plasma, liver and tumour xenografts*

Capecitabine and metabolite concentrations were determined both in plasma and tissues according to our previously published method.[20] Briefly, 50 mg of tissue was homogenised with 250 μl of 50 mM ammonium acetate:acetonitrile (1:3 v/v). After centrifugation at 3500 g for 10 min at 4 °C, the supernatant was transferred into a 200 μl tapered well 96-well microplate and evaporated to dryness. The dried extract was resuspended in 100 μl water and 10 μl was analysed by HPLC. For plasma samples, 50 μl of plasma was mixed with 150 μl of acetonitrile and processed similarly. Compounds were separated on a Develosil ODS-UG-3 column (4.6 × 150 mm, 3 μm) (Nomura Chemical) and mass spectrometry analysis was carried out on a Finnigan TSQ Quantum Discovery mass spectrometer using specific SRM transitions for each compound. Results were expressed as means ± SD values for three samples for each tissue.

2.6. *Statistical analysis*

For the cytotoxicity assays, growth inhibition curves were plotted as a percentage of control cells and IC_{50} estimates were determined by Graphpad Prism 3 Software (Graphpad Software, San Diego, CA) using a sigmoidal curve fitting with variable slope. The goodness of fit determined by r^2 was greater than 0.9 and the Hill coefficient <−1.

The gene expression data analysis of xenograft samples used a one-way ANOVA and Bonferroni multiple comparisons between controls and treated samples. The normality of the distributions was verified prior to the ANOVA.

3. Results

3.1. *Gene expression profiles of capecitabine-metabolising enzymes differ in tumour xenografts, mouse liver and human liver*

In vivo, the initial metabolism of capecitabine takes place in the liver. The level of expression of capecitabine-metabolising enzymes might influence the extent of capecitabine conversion into its active metabolites and therefore which metabolite is delivered to the tumour. A gene expression analysis of capecitabine metabolising enzymes was carried out in mouse liver, HCT 116 colon cancer xenografts and fresh human hepatocytes by qRT-PCR according to a previously published method.[17]

There was a significantly different pattern in gene expression between mouse liver and HCT 116 xenografts (Fig. 1). CES1 and DPD were the most prominent enzymes expressed in mouse liver tissue compared to HCT 116 xenografts (CES1 was not expressed in HCT 116 xenografts). Conversely, the level of CDD gene expression was ~100-fold lower in mouse liver compared to HCT 116 xenografts (GOI/REF = 0.02 and 3.05, respectively). UP expression was similar in mouse liver and HCT 116 xenografts (GOI/REF = 0.45 and 0.62, respectively). TP expression was higher in tumour xenografts (mean GOI/REF = 1.65) than in the mouse liver (mean GOI/REF = 0.6) while DPD expression was ~100 times greater in mouse liver than in HCT 116 xenografts. Overall, the TP/DPD ratios ranged from 0.005 to 0.03 in liver and 5.02–5.36 in HCT 116 xenografts.

To identify potential interspecies differences in drug metabolism, the gene expression was compared between mouse liver and human hepatocytes. The variability between donors was relatively limited with the coefficient of variation ranging from 26% for DPD to 58% for CES1. The major differences in gene expression were observed for CES2 and CDD: CES2 expression was ~100-fold higher in human liver than in mouse liver tissues (GOI/REF = 17 and 0.1, respectively). A similar difference was observed for the expression of CDD (GOI/REF = 2.5 and 0.02, respectively). The consequence of these differences is potentially a greater conversion of capecitabine to 5′DFCR and subsequently to 5′DFUR in human liver, in comparison to mouse liver.

3.2. *CDD, TP and DPD expressions influence both 5′DFCR and 5′DFUR cytotoxicities*

The growth inhibitory effect of capecitabine and metabolites is linked in part to the metabolism of the drug in cancer cells.

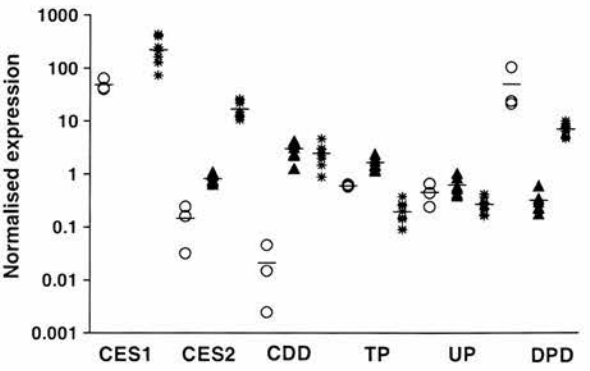


Fig. 1 – Gene expression of capecitabine metabolising enzymes in mouse liver and HCT 116 xenografts. Normalised gene expression was determined by quantitative RT-PCR for CES1, CES2, CDD, TP and DPD in mouse liver (○), HCT 116 xenografts (▲) and human hepatocytes (✳). Note that CES1 was not expressed in HCT 116 xenografts.

Table 1 – Cytotoxicity of capecitabine and metabolites in colon cancer cell lines

	IC_{50} values (μM)							
	CAPE (mean ± SD)		5′-Deoxy-5-fluorocytidine (5′DFCR mean ± SD)		5′-Deoxy-5-fluorouridine (5′DFUR, mean ± SD)		5-Fluorouracil (5-FU, mean ± SD)	
HCT 116	2850	995	57.5	4.80	35.2	2.00	7.70	0.700
HT29	1590	219	186	40.8	57.5	6.10	6.70	0.800
SW620	4190	1150	217	36.0	57.5	6.60	22.3	2.70
HCT8	5970	4200	121	20.0	66.8	11.2	6.70	0.70
HCT15	5840	3970	586	82.0	86.0	12.0	9.90	1.30
COLO205	863	165	59.9	10.0	32.2	7.00	4.90	0.60

The cytotoxic effect of 24-h exposure to capecitabine, 5′DFCR, 5′DFUR and 5-FU was determined by SRB assay. Results are mean ± SD of 3–5 experiments performed in triplicate.

To identify potentially limiting steps in capecitabine metabolism in cells, the cytotoxicity of capecitabine and its metabolites was determined in six colon cancer cell lines using the SRB assay and compared with the gene expression profile of capecitabine-metabolising enzymes in the same cell lines determined by qRT-PCR.

Capecitabine induced a significant cytotoxic effect *in vitro* only at high concentrations (Table 1). Mean IC_{50} values varied from 860 μM in COLO205 cells to 6000 μM in HCT8 cells. Both 5′DFCR and 5′DFUR were significantly more cytotoxic than capecitabine: IC_{50} values ranged from 58 to 590 μM for 5′DFCR and 32 to 86 μM for 5′DFUR. The distribution of IC_{50} estimates for 5′DFCR was greater across the six cell lines (10-fold range) than for 5′DFUR (<3-fold range). Finally, 5-FU IC_{50}s varied 4.5-fold between the most sensitive (COLO205) and least sensitive (SW620) cell lines.

Gene expression analysis of capecitabine-metabolising enzymes, i.e. CES1 and 2, CDD, TP, DPD was also carried out using qRT-PCR (Table 2). CES1 was significantly expressed only in HCT8 and COLO205 cells (normalised CES1 = 1.29 and 1.24, respectively). CDD expression varied 10-fold across the cell lines, COLO205 expressing the highest levels (normalised CDD = 2.54) and HCT-15 the lowest (normalised CDD = 0.22). TP was expressed significantly only in HCT 116 and HT29 cells (normalised TP = 0.38 and 0.33, respectively). Finally, both HT29 and HCT-15 cells express high levels of DPD (normalised DPD = 0.93 and 1.19, respectively). Overall, only HCT 116 and HT29 cell lines expressed significant levels of all the enzymes involved in the metabolic pathway of capecitabine.

Table 2 – Gene expression analysis of capecitabine metabolising enzymes in colon cancer cell lines

	CES1	CES2	CDD	TP	DPD
HCT 116	<LOQ	0.30	1.05	0.38	0.13
HT29	<LOQ	0.53	0.25	0.33	0.93
SW620	0.02	1.27	0.28	<LOQ	<LOQ
HCT8	1.29	0.89	1.78	<LOQ	0.01
HCT15	<LOQ	0.61	0.22	<LOQ	1.19
COLO205	1.24	0.31	2.54	0.05	<LOQ

CES1, CES2, CDD, TP and DPD mRNA expressions were determined by qRT-PCR as previously published [Macpherson, 2006 #178]. Gene expressions are normalised by the geometric mean of 18S, B2M, GAPDH and POLR2 reference genes. Results are mean ± SD of 2 independent experiments performed in triplicate. <LOQ: values lower than limit of quantification.

When the ratios of 5′DFCR and 5′DFUR IC_{50} estimates were plotted against the mRNA expression of CDD, cell lines which displayed low expression (<1.5) had a high ratio (>2) suggesting that conversion of 5′DFCR within the tumour cells was a major factor determining 5′DFCR cytotoxic effect (Fig. 2A). Moreover, the difference in 5′DFUR IC_{50} estimates between cell lines was unrelated to TP expression. However, the ratio of TP and DPD expressions did correlate with 5′DFUR IC_{50} (Fig. 2B): Four cell lines expressing TP/DPD ratios lower than 0.6 displayed significantly higher 5′DFUR IC_{50} values (>50 μM) than the two cell lines presenting ratios greater than 3. However, the IC_{50} values for these two cell lines were very similar (~33 μM) despite a ~6-fold difference in the ratios. No correlation was observed between UP or UP/DPD and 5′DFUR cytotoxicity.

3.3. *Difference in gene expression is consistent with pharmacokinetic profiles in plasma, liver and tumour xenografts*

To establish whether the differences in gene expression would impact on metabolism *in vivo*, a pharmacokinetic/pharmacodynamic study was carried out in mice bearing HCT 116 xenografts receiving 0.52 and 2.1 mmol/kg/d of capecitabine by oral gavage. Capecitabine administered at 0.52 mmol/kg/day induced partial control of HCT 116 xenografts tumour growth: growth rate = 7.5 ± 0.5 on day 21 (Fig. 3A). Capecitabine 2.1 mmol/kg/day achieved complete control of tumour growth during the treatment period: growth rate = 1 ± 0.2 on day 21 (this dose of capecitabine is similar to the dose used in patients).

The pharmacokinetic study showed that 5′DFCR was the most abundant metabolite in both plasma (Fig. 3B) and liver (Fig. 3C): C_{max} = 110 ± 10 μM in plasma and 590 ± 140 μM in liver. When considering the concentrations 0–8 h after administration, there was a significant correlation between liver and plasma concentrations of 5′DFCR at both dose levels (r^2 = 0.91, n = 18 and r^2 = 0.95, n = 18 at 0.52 and 2.1 mmol/kg/day of capecitabine, respectively). The low CDD expression observed in mouse liver could therefore be a limiting factor to the conversion of 5′DFCR in the liver.

The residual concentrations determined in liver and tumour tissues on day 7 (Fig. 3D), 72 h after the previous oral

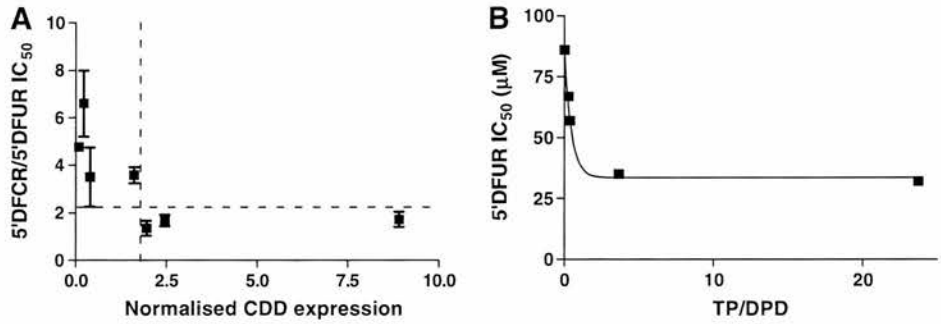


Fig. 2 – Impact of CDD and TP/DPD on capecitabine metabolites cytotoxicity. (A) The ratio of IC_{50} values for 5′DFCR and 5′DFUR is plotted against CDD mRNA expression in six colon cancer cell lines. (B) Correlation between TP/DPD ratios and cytotoxicity of 5′DFUR.

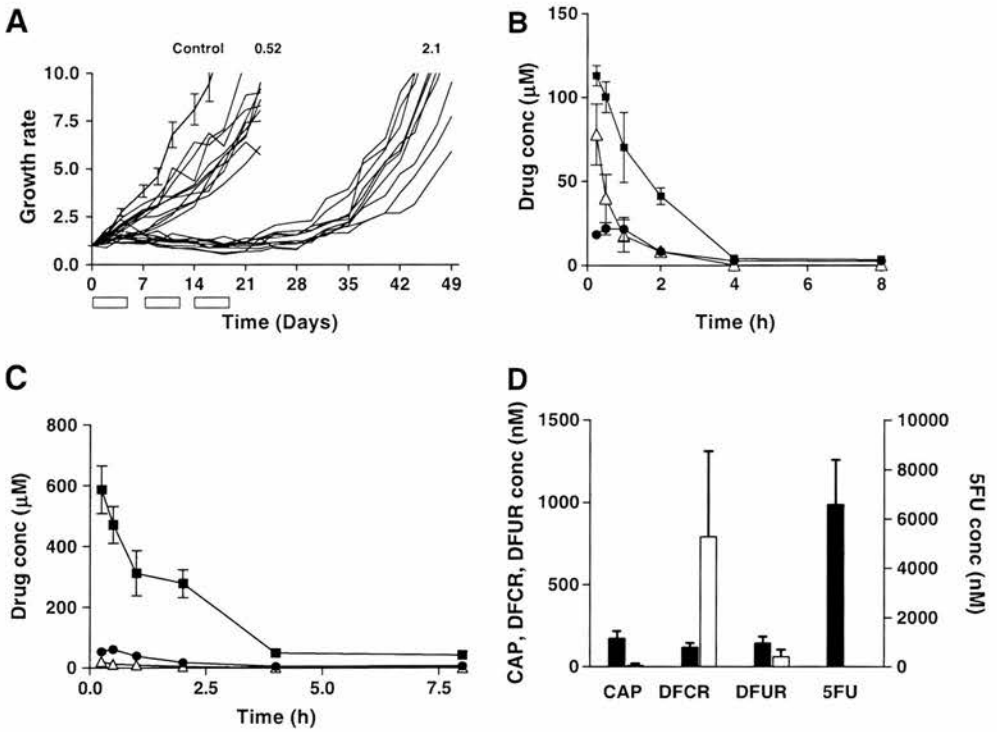


Fig. 3 – Pharmacokinetic/pharmacodynamic study with capecitabine. (A) capecitabine efficacy in HCT 116 xenografts. HCT 116 cells were implanted subcutaneously in nude mice. Animals received either capecitabine 0.52 or 2.1 mmol/kg once daily by oral gavage for consecutive 5 days/week for 3 weeks (days 0–4, 7–11, 14–18). The growth rate is presented for each individual xenograft. (B) Capecitabine and metabolite concentrations in plasma. (C) Liver tissue after the first dose of capecitabine (2.1 mmol/kg); capecitabine (△), 5′DFCR (■), 5′DFUR (●). (D) Concentrations of capecitabine (CAP), 5′DFCR, 5′DFUR and 5-FU in liver tissue (white bars) and HCT 116 xenografts (black bars) on day 7 of treatment.

dose of capecitabine (2.1 mmol/kg/day) administered on day 4, were unexpected, demonstrating high concentrations of 5′DFCR in the liver (790 nM). 5′DFCR was also demonstrable in the tumour, albeit at a lower concentration (120 nM) than in the liver. Also, surprisingly high concentrations of 5-FU were present in the tumour (mean 6600 nM) at this time-point, whilst 5-FU was undetectable in either the liver or the plasma. 5-FU was also detectable in the tumours of animals treated with capecitabine 0.52 mmol/kg/day: 2200, 4000 and 4800 nM (mean values on days 7, 14 and 21, respectively (data not shown)).

A gene expression analysis was carried out in tumour xenografts during the course of the treatment. TP/DPD increased over time in xenografts exposed to 2.1 mmol/kg/day of capecitabine: From 6.6 on day 7 to 10 on day 21 while xenografts exposed to 0.52 mmol/kg/day of capecitabine increased slightly from 3.1 on day 7 to 5.3 on day 21. There was a significant correlation between tumour 5-FU concentrations (days

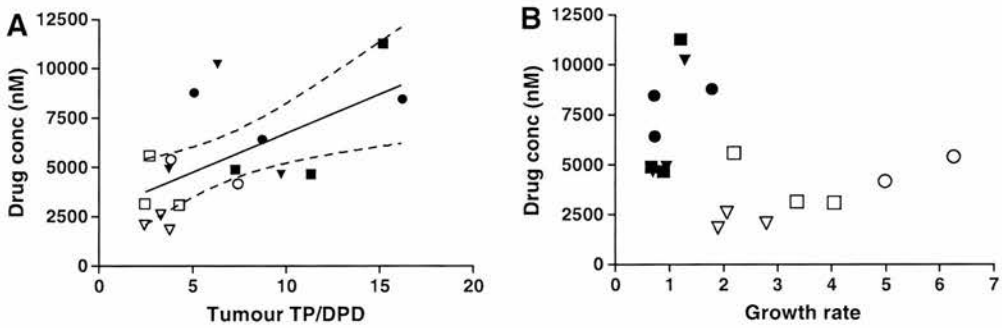


Fig. 4 – Pharmacokinetic/pharmacodynamic relationships after administration of 0.52 mmol/kg/d (open symbols) and 2.1 mmol/kg/d (close symbols) of capecitabine. Samples were collected on day 7 (▽, ▼), day 14 (□, ■) and day 21 (○, ●) after administration. (A) Correlation between 5-FU concentrations in xenograft tumours and TP/DPD ratios. The correlation line and 95% confidence intervals are presented. (B) 5-FU concentrations in xenograft tumours and tumour response. Individual xenografts data are presented.

7, 14 and 21 at both dose levels) and TP/DPD gene expression ($r^2 = 0.37$, $p = 0.01$, $n = 16$) (Fig. 4A). No other correlations were observed with 5′DFCR, 5′DFUR or capecitabine. In addition, no correlation was observed between TP or UP expression and either capecitabine or metabolite concentrations.

Moreover, 5-FU concentrations were significantly related to tumour response (Fig. 4B): Xenografts exposed to 2.1 mmol/kg/d of capecitabine leading to 5-FU concentrations >4000 nM showed constant control of tumour growth (growth rate <2 at all time). Conversely, xenografts exposed to 0.52 mmol/kg/d of capecitabine with lower tumour 5-FU concentrations showed a progressive increase in growth rate over time. Even when 5-FU concentrations reached 4000 nM on day 21, the growth rate was high. This suggests that early control of tumour growth with high concentrations of 5-FU in the tumour is necessary for growth control.

4. Discussion

Capecitabine undergoes extensive metabolism in both liver and tumour. The studies presented here were undertaken to test the hypothesis that gene expression analysis between different target tissues could help to predict interspecies differences, potential variability between patients and tumour response.

The gene expression data presented in this study are consistent with enzymatic activities previously reported by Tsukamoto et al., in particular the low expression of CDD in mouse liver and its greater enzymatic activity in human compared to mouse liver.[21] Similar correlations were previously shown for DPD, TS and TP, allowing the use of quantitative RT-PCR (qRT-PCR) as an indirect measure of enzyme activities.[22]

In *in vitro* studies determining the growth inhibition of capecitabine and metabolites in six colon cancer cell lines, CDD expression correlated with 5′DFCR cytotoxicity, consistent with previously published data.[11] Cell lines demonstrating high TP/DPD ratios also showed greater sensitivity to 5′DFUR.

Differential gene expression between mouse liver and HCT 116 xenografts was able to explain the different pharmacokinetic profiles for capecitabine and metabolites in plasma, liver and xenografts: high carboxylesterase expression in the liver facilitated the efficient conversion of capecitabine to 5′DFCR, consistent with low capecitabine concentrations in the liver compared to 5′DFCR concentrations. However, the very low level of hepatic CDD in the mouse liver became a limiting factor in 5′DFCR conversion, resulting in higher concentrations of 5′DFCR than 5′DFUR in the plasma. High DPD expression in mouse liver in conjunction with relatively low TP expression explained the absence of 5-FU in the liver, as any 5-FU which was formed would have been rapidly converted to dihydro-5-FU and other metabolites. In HCT 116 xenografts, the absence of CES led to significant concentrations of capecitabine in the tumour tissue. Concentrations of 5′DFUR were higher than 5′DFCR due to high CDD expression. As previously observed by Ishikawa et al., 5-FU was the predominant metabolite in tumour.[12] In contrast to the situation in liver, high TP expression associated with low DPD expression favoured 5-FU formation over its detoxification in this particular xenograft model.

The low efficiency of 5′DFUR conversion in mouse liver by both UP and TP observed in this study was first identified by El-Kouni et al.[23] The same study showed that human hepatic TP and not UP was the main enzyme converting 5′DFUR in liver. More recently, UP knock-out mice, which present high plasma and tissue uridine concentrations, were shown to tolerate 5-FU (85 mg/kg) better than wild type animals, suggesting that UP plays an important role in the anabolism of 5-FU.[24] However, neither 5′DFUR nor capecitabine was tested in that study. In tumours and cell lines, a large body of literature focuses on TP as a determinant for tumour-specific formation of 5-FU after capecitabine administration.[12,13]However, uridine phosphorylase can convert 5′DFUR into 5-FU in human and mouse tumour cell lines.[25] In this study, TP expression was ~3-fold higher than UP in HCT 116 xenografts but no correlation was observed between TP or UP expression and 5′DFUR cytotoxicity. However, *in vitro*, cell lines presenting high TP/DPD ratios showed a greater sensitivity to 5′DFUR. Moreover, *in vivo*, 5-FU concentrations in the tumour also correlated with TP/DPD expression. This is consistent with results from previous pre-clinical studies using human cancer

xenografts and correlating TP/DPD ratios with the efficacy of capecitabine and 5′DFUR.[26]

The gene expression analysis identifies CDD as an important factor for the delivery of 5′DFUR to the tumour. The greater CDD expression observed in fresh human hepatocytes as compared to mouse liver predicts the greater conversion of 5′DFCR into 5′DFUR. Higher concentrations of 5′DFUR were indeed reported by Mader et al. in human plasma.[27] This confirmed the potential role of CDD in clinical outcome following capecitabine administration. CDD polymorphisms induce different metabolic activity and their potential importance in relation to capecitabine therapy merits further study.[28]

These studies have confirmed the importance of the expression of drug metabolising enzymes in the pharmacokinetics and activity of this commonly used agent. TP/DPD is a predictive marker of sensitivity and its variations over time, although important, are related to tumour 5-FU concentrations. Whilst the invasive assessment of gene expression and fluoropyrimidine PK in normal liver and metastatic cancer tissue using sequential biopsies in patients is not feasible, advances in non-invasive imaging using 19F imaging using magnetic resonance spectroscopy might allow a more detailed monitoring of capecitabine PK in individual patients. A recent study by Klomp et al.[29] has demonstrated the feasibility of such an approach.

Conflict of interest statement

Prof. Jodrell is a member of the editorial board of the Xeloda website (Roche Pharmaceuticals) and member of the Data Monitoring and Safety Board for CHAT study (Honoraria paid). Prof. Jodrell has received departmental support for clinical trials with capecitabine (Roche Pharmaceuticals).

Acknowledgements

This work has been funded by the Cancer Research UK Programme Grant #A5008. We are grateful to Roche Pharmaceuticals for providing capecitabine, 5′DFCR, 5′DFUR and 5-FU for both the *in vitro* and *in vivo* studies.

REFERENCES

1. Hoff PM, Ansari R, Batist G, Cox J, Kocha W, Kuperminc M, et al. Comparison of oral capecitabine versus intravenous fluorouracil plus leucovorin as first-line treatment in 605 patients with metastatic colorectal cancer: results of a randomized phase III study. *J Clin Oncol* 2001;**19**(8):2282–92.
2. Scheithauer W, McKendrick J, Begbie S, Borner M, Burns WI, Burris HA, et al. Oral capecitabine as an alternative to i.v. 5-fluorouracil-based adjuvant therapy for colon cancer: safety results of a randomized, phase III trial. *Ann Oncol* 2003;**14**(12):1735–43.
3. Twelves C, Wong A, Nowacki MP, Abt M, Burris 3rd H, Carrato A, et al. Capecitabine as adjuvant treatment for stage III colon cancer. *New Engl J Med* 2005;**352**(26):2696–704.
4. Quinney SK, Sanghani SP, Davis WI, Hurley TD, Sun Z, Murry DJ, et al. Hydrolysis of capecitabine to 5′-deoxy-5-fluorocytidine by human carboxylesterases and inhibition by loperamide. *J Pharmacol Exp Ther* 2005;**313**(3):1011–6 [Epub 2005 Feb 1].
5. Reigner B, Blesch K, Weidekamm E. Clinical pharmacokinetics of capecitabine. *Clin Pharmacokinet* 2001;**40**(2):85–104.
6. Wagstaff AJ, Ibbotson T, Goa KL. Capecitabine: a review of its pharmacology and therapeutic efficacy in the management of advanced breast cancer. *Drugs* 2003;**63**(2):217–36.
7. Tsukamoto Y, Kato Y, Ura M, Horii I, Ishitsuka H, Kusuhara H, et al. A physiologically based pharmacokinetic analysis of capecitabine, a triple prodrug of 5-FU, in humans: the mechanism for tumor-selective accumulation of 5-FU. *Pharm Res* 2001;**18**(8):1190–202.
8. Gieschke R, Burger HU, Reigner B, Blesch KS, Steimer JL. Population pharmacokinetics and concentration–effect relationships of capecitabine metabolites in colorectal cancer patients. *Br J Clin Pharmacol* 2003;**55**(3):252–63.
9. Gieschke R, Reigner B, Blesch KS, Steimer JL. Population pharmacokinetic analysis of the major metabolites of capecitabine. *J Pharmacokinet Pharmacodyn* 2002;**29**(1):25–47.
10. Schuller J, Cassidy J, Dumont E, Roos B, Durston S, Banken L, et al. Preferential activation of capecitabine in tumor following oral administration to colorectal cancer patients. *Cancer Chemother Pharmacol* 2000;**45**(4):291–7.
11. Morita T, Matsuzaki A, Kurokawa S, Tokue A. Forced expression of cytidine deaminase confers sensitivity to capecitabine. *Oncology* 2003;**65**(3):267–74.
12. Ishikawa T, Utoh M, Sawada N, Nishida M, Fukase Y, Sekiguchi F, et al. Tumor selective delivery of 5-fluorouracil by capecitabine, a new oral fluoropyrimidine carbamate, in human cancer xenografts. *Biochem Pharmacol* 1998;**55**(7):1091–7.
13. Miwa M, Ura M, Nishida M, Sawada N, Ishikawa T, Mori K, et al. Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. *Eur J Cancer* 1998;**34**(8):1274–81.
14. Ninomiya I, Terada I, Yoshizumi T, Takino T, Nagai N, Morita A, et al. Anti-metastatic effect of capecitabine on human colon cancer xenografts in nude mouse rectum. *Int J Cancer* 2004;**112**(1):135–42.
15. de Bruin M, van Capel T, Van der Born K, Kruyt FA, Fukushima K, Hoekman K, et al. Role of platelet-derived endothelial cell growth factor/thymidine phosphorylase in fluoropyrimidine sensitivity. *Brit J Cancer* 2003;**88**(6):957–64.
16. Adlard JW, Richman SD, Seymour MT, Quirke P. Prediction of the response of colorectal cancer to systemic therapy. *Lancet Oncol* 2002;**3**(2):75–82.
17. Macpherson J, Jodrell D, Guichard S. Validation of real-time reverse-transcription-polymerase chain reaction for quantification of capecitabine-metabolising enzymes. *Anal Biochem* 2006;**350**(1):71–80.
18. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990;**82**(13):1107–12.
19. Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, et al. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia 2nd ed. *Brit J Cancer* 1998;**77**:1–10.
20. Guichard S, Mayer I, Jodrell D. Simultaneous determination of capecitabine and its metabolites by HPLC and mass spectrometry for preclinical and clinical studies. *J Chromatogr B* 2005;**826**(1–2):232–7.
21. Tsukamoto Y, Kato Y, Ura M, Horii I, Ishikawa T, Ishitsuka H, et al. Investigation of 5-FU disposition after oral administration of capecitabine, a triple-prodrug of 5-FU, using a physiologically based pharmacokinetic model in a human

cancer xenograft model: comparison of the simulated 5-FU exposures in the tumour tissue between human and xenograft model. *Biopharm Drug Dispos* 2001;**22**(1):1–14.
22. Eliason JF, Megyeri A. Potential for predicting toxicity and response of fluoropyrimidines in patients. *Curr Drug Targets* 2004;**5**(4):383–8.
23. el Kouni MH, el Kouni MM, Naguib FN. Differences in activities and substrate specificity of human and murine pyrimidine nucleoside phosphorylases: implications for chemotherapy with 5-fluoropyrimidines. *Cancer Res* 1993;**53**(16):3687–93.
24. Cao D, Leffert JJ, McCabe J, Kim B, Pizzorno G. Abnormalities in uridine homeostatic regulation and pyrimidine nucleotide metabolism as a consequence of the deletion of the uridine phosphorylase gene. *J Biol Chem* 2005;**280**(22):21169–75 [Epub 2005 Mar 16].
25. Peters GJ, Laurensse E, Leyva A, Lankelma J, Pinedo HM. Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5′-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. *Cancer Res* 1986;**46**(1):20–8.
26. Ishikawa T, Sekiguchi F, Fukase Y, Sawada N, Ishitsuka H. Positive correlation between the efficacy of capecitabine and doxifluridine and the ratio of thymidine phosphorylase to dihydropyrimidine dehydrogenase activities in tumors in human cancer xenografts. *Cancer Res* 1998;**58**(4):685–90.
27. Mader RM, Schrolnberger C, Rizovski B, Brunner M, Wenzel C, Locker G, et al. Penetration of capecitabine and its metabolites into malignant and healthy tissues of patients with advanced breast cancer. *Br J Cancer* 2003;**88**(5):782–7.
28. Kirch HC, Schroder J, Hoppe H, Esche H, Seeber S, Schutte J. Recombinant gene products of two natural variants of the human cytidine deaminase gene confer different deamination rates of cytarabine in vitro. *Exp Hematol* 1998;**26**(5):421–5.
29. Klomp D, van Laarhoven H, Scheenen T, Kamm Y, Heerschap A. Quantitative (19)F MR spectroscopy at 3 T to detect heterogeneous capecitabine metabolism in human liver. *NMR Biomed* 2007;**20**(5):485–92.